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UNIVERSITY OF LONDON

**Sub-cellular localization and regulatory
mechanisms of AMPA receptors in motor neurons
of a murine model of
familial Amyotrophic Lateral Sclerosis.**

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Thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease leading to progressive muscle wasting and paralysis and premature death. The disease is predominantly sporadic (sALS) in nature with approximately 10% of case inherited (familial ALS). In the present study, I have examined the subcellular distribution and expression of the AMPA receptor subunits and the proteins involved in their trafficking between the cytosol and postsynaptic membrane (ABP, PICK1 and NSF) in a mouse model of familial ALS (SOD1G93A mice) at different stages of the disease.

The main finding was a decrease of GluR2 AMPA receptor subunit selectively in cytosol, but not in synaptic membrane of spinal motor neurons of SOD1G93A mice before symptoms onset. This suggests that an impairment in the usually well-organized trafficking of the GluR2 AMPA is likely the cause of the increased degradation of this receptor subunit. In support of this, increased level of the trafficking protein NSF, able to increase the delivery of GluR2 at the cell surface, was reported selectively in the motor neurons at the presymptomatic stage of the disease in SOD1G93A mice.

Since GluR2 plays an important role in regulating the calcium entrance in the cells by the AMPA receptors, I hypothesise that the increase of NSF may be an attempt to protect motor neurons from excitotoxicity by maintaining the sufficient levels of GluR2 subunit in the membrane. However, since an overall degradation of this subunit occurs in the motor neurons, this effect is probably no longer efficacious.

At symptomatic and end stage of the disease increased immunoreactivity of GluR1, 2 and 3 AMPA receptor subunits was found in the activated glial cells. Although there are

evidences of a role of reactive glia in favouring the neuronal excitotoxicity, the relevance of this effect needs to be investigated.

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LIST OF ABBREVIATIONS

ABP	AMPA Binding Protein
ALS	Amyotrophic Lateral Sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
CDK5	Cyclin-Dependent Kinase 5
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic Acid
EAAT	Excitatory Amino Acid Transporter
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
EMG	Electromyography
FALS	Familial ALS
GABA	γ -amino butyric acid
GFAP	Glial Fibrillary Acidic Protein
GluR	Glutamate receptor
HEPES	4-(2-Hydroxyethyl)piperazine-1- ethanesulfonic acid
HRP	Horseradish peroxidase
hSOD1	Human SOD1
IGF	Insulin growth factor
IgG	Immunoglobulin G
iNOS	Inducible nitric oxide synthase

KA	Kaininc Acid
KDa	Kilodaltons
Kb	Kilobases
LMN	Lower motor neurones
Loa	Legs-at-odd-angles
MAPK	Mitogen Activated Protein Kinase
MND	Motor Neurone Disease
mRNA	Messenger RNA
mSOD1	Murine SOD1
NAIP	Neuronal apoptosis inhibitory protein
NBQX	6-nitro-7-sulfamobenzoquinoxaline-2,3-dione
NF-H	High molecular weight neurofilament
NF-L	Low molecular weight neurofilament
NGS	Normal goat serum
NMDA	The <i>N</i> -methyl-D-aspartate
NO	Nitric Oxide
NSF	N-ethylmaleimide sensitive fusion protein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDC	<i>L-trans</i> -2,4-Pyrrolidine Dicarboxylate
PICK1	Protein Interacting with C-Kinase 1
PKA	Protein Kinase A
PKC	Protein Kinase C
PLS	Primary Lateral Sclerosis

PMN	Progressive Motor Neuronopathy
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
S.E.M	Standard error of the Mean
SOD1	Superoxide dismutase 1
TBS	Tris buffered saline
TBST	TBS + 0,1% Tween-20
SOD1G93A	Superoxide dismutase 1 with G93A mutation
Tris	Tris(hydroxymethyl)methylamine
UMN	Upper motor neurones
VEGF	Vascular endothelial growth factor

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I dedicate this Ph.D. thesis to Francesco, Lina and Marisa.

CHAPTER 1

GENERAL INTRODUCTION

1.1 CLASSIFICATION OF MOTOR NEURON DISORDERS

The motor neuron diseases (MND) are a group of progressive neurological disorders that destroy motor neurons, the cells that control voluntary muscle activity. Different MND have been classified so far that comprises disorders of lower or the upper motor neurons, called spinal muscular atrophy, progressive muscular atrophy, progressive and primary lateral sclerosis, and amyotrophic lateral sclerosis.

1.1.1 Spinal Muscular Atrophy (SMA):

Is a term applied to a number of different disorders, all having in common a genetic cause and the manifestation of weakness due to loss of the motor neurons of the spinal cord and brainstem. The most common form of SMA is caused by mutation of the SMN (Survival Motor Neuron) gene. There are two copies of the gene SMN, SMN1 and SMN2. SMA is caused by loss of the SMN1 gene from both chromosomes (Monani et al, 2005). SMA manifests over a wide range of severity affecting infants through adults. The spectrum of severity of SMA has been divided arbitrarily into three groups by the level of weakness and is partly related to how well the remaining SMN2 genes can compensate for the loss of SMN1.

- **Infantile SMA - Type 1 or Werdnig-Hoffmann disease** (generally 0-6 months). SMA type 1, also known as severe infantile SMA or Werdnig

Hoffmann disease, is the most severe, and manifests in the first year of life with the inability to ever maintain an independent sitting position.

- **Intermediate SMA - Type 2** (generally 7-18 months). Type 2 SMA, or intermediate SMA, describes those children who are never able to stand and walk, but who are able to maintain a sitting position at least some time in their life. The onset of weakness is usually recognized some time between 6 and 18 months.
- **Juvenile SMA - Type 3 or Kugelberg-Welander disease** (generally >18 months). SMA type 3 describes those who are able to walk at some time. It is also known as Kugelberg Welander disease.

1.1.2 Progressive bulbar palsy

It is a form of motor neuron disease characterized by dysfunction of the muscles controlled by the cranial nerves of the lower brain stem specifically, the glossopharyngeal nerve (IX), vagus nerve (X), and hypoglossal nerve (XII).

1.1.3 Primary Lateral Sclerosis (PLS)

It is a rare neuromuscular disease characterized by progressive muscle weakness in the voluntary muscles. Onset of PLS usually occurs after age 50. Symptoms may include difficulty with balance, weakness and stiffness in the legs, and clumsiness. Other symptoms may include spasticity (sudden, involuntary muscle spasms) in the hands, feet, or legs; foot dragging, and speech problems due to involvement of the facial muscles. The disorder usually begins in the legs, but it may also start in the tongue or the hands.

The disease, not hereditary, progresses gradually over a number of years, or even decades. In PLS, there is no evidence of the degeneration of spinal motor neurons or muscle wasting (amyotrophy) that occurs in amyotrophic lateral sclerosis, which it resembles. Diagnosis of PLS is often delayed because it is mistaken for ALS.

1.1.4 Amyotrophic Lateral Sclerosis (ALS)

ALS is the most common among MND. J. M. Charcot in 1874 first described the clinical and neuropathological features of ALS among a heterogeneous group of MND. The origin of the term “ALS” arises from the post mortem pathological analysis of patients describing the loss of muscular mass (amyotrophic) associated with the hardening (sclerosis) of the lateral columns of the spinal cord. So far, an early diagnosis of the pathology by a specific marker is not possible and the following clinical signs are the only way to diagnose ALS pathology:

1.2 DIAGNOSIS OF THE AMYOTROPHIC LATERAL SCLEROSIS

ALS is a devastating disease affecting the upper and lower motor neurons which leads to a progressive muscular weakness until a complete motor paralysis and death within 3-5 years from the diagnosis. So far, an early diagnosis of the pathology by a specific marker is not possible and the clinical signs are the only mode to diagnose ALS pathology. The requirements for diagnosis of ALS were defined by the El Escorial criteria in 1994 and subsequently revised in order to increase their sensitivity by a workshop convened at Airlie Conference Center, Warrenton, Virginia on April 2-4, 1998 by the World Federation of Neurology Research Committee on Motor Neuron Diseases.

According to these criteria the diagnosis of ALS requires the presence of:

-signs of *lower motor neuron (LMN) degeneration* by clinical, electrophysiological or neuropathologic examination

-evidence of *upper motor neuron (UMN) degeneration* by clinical examination.

-*progressive spread of symptoms or signs* within a region or to other regions, as determined by history or examination,

together with the absence of:

-electrophysiological and pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and

- neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

A careful physical and neurological examination must search for clinical evidence of UMN and LMN signs (Table 1.0) in four regions (brainstem, cervical, thoracic or lumbosacral spinal cord) of the central nervous system (CNS). Tests should be applied, as clinically indicated, to exclude other disease processes. These should include electrodiagnostic, neurophysiological, neuroimaging and clinical laboratory studies. The clinical diagnosis of ALS, without pathological confirmation, may be categorized into various levels of certainty.

According to clinical criteria it is possible to define four diagnostic categories:

Definite ALS

It is defined on clinical evidence alone by the presence of UMN, as well as LMN signs, in the bulbar region and at least two of the other spinal regions (cervical, thoracic and lumbosacral).

Probable ALS

It is defined on clinical evidence alone by the presence of UMN and LMN signs in at least two regions with some UMN signs necessarily rostral to the LMN signs.

Probable - Laboratory-supported ALS

It is defined when clinical signs of UMN and LMN dysfunction are in only one region, or when UMN signs alone are present in one region, and LMN signs defined by EMG criteria are present in at least two limbs, with proper application of neuroimaging and clinical laboratory protocols to exclude other causes.

Possible ALS

It is defined when clinical signs of UMN and LMN dysfunction are found in only one region or UMN signs alone are found in two or more regions; or LMN signs are rostral to UMN signs

Suspected ALS

It manifests only with LMN signs in two or more regions which is typical also of the progressive muscular atrophy. Therefore, this category has been deleted from the revised El Escorial Criteria for the diagnosis of ALS

	<i>Lower motor neurons</i>	<i>Upper motor neurons</i>
<i>Symptoms</i>	Fatigue Weakness Cramps Twitching of muscles Incoordination	Weakness Incoordination Stiffness Slowing of distal movement
<i>Signs</i>	Weakness Fasciculations Suppression of reflexes Hypotonia Atrophy	Spasticity Brisk reflexes Babinski and Hoffman signs Weakness Pseudobulbar effect

Table 1.0: Lower and upper motor neuron dysfunctions in ALS

1.3 CLINICAL PHENOTYPE OF AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis is characterized by a sequence of symptoms and rate progression that varies from person to person. The degeneration of the UMNs lead to development of symptoms represented by muscle weakness, spasticity, and hyperreflexia; while the loss of LMNs determines the muscle weakness, atrophy, fasciculations, hypotonia, and areflexia. Since ALS affects only motor neurons, the senses of sight, touch, hearing, taste and smell, muscles of the eyes and bladder are generally not affected.

The initial symptoms of the disease are so slight that they are frequently overlooked and can be quite variable in different people. Also the rate of disease progression can be quite variable from one patient to another. With regard to the appearance of symptoms and the progression of the illness, the course of the disease may include the following:

- muscle weakness in one or more of the following: hands, arms, legs or the muscles of speech, swallowing or breathing
- twitching (fasciculation) and cramping of muscles, especially those in the hands and feet
- impairment of the use of the arms and legs
- 'thick speech' and difficulty in projecting the voice
- in more advanced stages, shortness of breath, difficulty in breathing and swallowing

ALS symptoms may be related to either *limb onset*, *bulbar onset* or both.

Limb onset affects the extremities and torso. A general weakness of one or more legs, arms and hands and fatigue may be the first sign of the disease. Muscle cramping and fasciculations (muscle twitching) will begin to develop. As the disease progresses, the patient may stumble while walking or lose grip of objects. Breathing may become laboured. Uncontrolled crying and/or laughing (pseudobulbar affect) can occur.

Bulbar onset affects the face, mouth and throat muscles. With bulbar onset, one's voice may begin to weaken and become slurred. Chewing and swallowing becomes difficult (dysphagia). Fasciculations will develop in the tongue. Limb onset cases will eventually develop bulbar symptoms.

Muscle weakness is a hallmark initial sign in ALS, occurring in approximately 60% of patients. Early symptoms vary with each individual, but usually include tripping, dropping things, abnormal fatigue of the arms and/or legs, slurred speech, muscle cramps and twitches and/or uncontrollable periods of laughing or crying. The hands and feet may be affected first, causing difficulty in lifting, walking or using the hands for the activities of daily living such as dressing, washing and buttoning clothes. As the weakening and paralysis continue to spread to the muscles of the trunk of the body, the disease eventually affects speech, swallowing, chewing and breathing. When the breathing muscles become affected, the patient will need permanent ventilation support in order to survive.

The progression rate scale of the pathology is based on two rating scale:

ALS Functional Rating Scale: Developed by the WFN (World Federation of Neurology) in 1996, the ALS Functional Rating Scale is a scorecard for assessing a patient's condition by looking at aspects such as swallowing, speaking, movement, etc. The score range is composed from 0 to 48, with 48 being normal and 0 being unable to perform.

Appel ALS Rating Scale: Based on much of the same aspects as the scale created by WFN. The AALS was developed at Baylor College of Medicine to provide a reliable, quantitative estimate of the functional impairment and symptom progression of patients with ALS (Appel et al., 1987; Haverkamp et al., 1995). It includes subjectively and objectively quantified assessments of:

-bulbar function (speech and swallowing)

-respiratory function

-muscle strength in arms and legs

-upper extremity function

-lower extremity function

The AALS total score is the sum of all 5 assessments and covers a broad range of disability. One important feature of this rating scale is that it provides a single quantitative index of clinical status that documents the variable manifestation and progression of ALS and predicts survival time in patients.

1.4 NEUROPATHOLOGY OF ALS

Combined upper motor neuron (UMN) and lower motor neuron (LMN) signs are involved in ALS and manifested by degenerative changes in the motor system. These changes have been documented since the studies of Charcot in the mid 19th century that revealed:

- a reduction in both the number and size of lower motor neurons in the ventral horns and in the bulbar motor nuclei
- myelin pallor in the corticospinal projection pathway (axonal loss degeneration)
- upper motor neuron soma alteration (much more variable)

1.4.1 Degeneration of Upper Motor Neuron (UMN)

There is variability in the autopsy of ALS about the neuropathological alterations of the UMN. The most severely affected cases showed a loss of the giant Betz cells in cortical layer (5° stratum) with an increased astrocytosis and microgliosis. However, it is not easy to determine a loss of UMN because there is no molecular marker that exclusively labels UMN. Furthermore, UMN degeneration leads to shrinkage of the cells so that Betz cells become indistinguishable from others cells in the cortical layers. Also, pallor of the corticospinal tract has been demonstrated in ALS patients as a consequence of the UMN degeneration. However, a different hypothesis suggests that the

axonopathy may be due to the dying back degeneration of the UMN axons rather than initial UMN cell bodies degeneration.

1.4.2 Degeneration of Lower Motor Neuron (LMN)

A selective loss of motor neurons in the spinal cord occurs in ALS. However, not all lower motor neurons undergo neurodegeneration. Two groups are spared in most cases: the sacral motor nucleus of Onufrowitz (Onuf's nucleus) which innervates muscles of the pelvic floor; and are responsible for maintaining retention of urinary and faecal continence, and the motor neurons that innervate the external ocular muscles (third, fourth and sixth cranial nerves) and preserve normal eye movement. The selective sparing of these groups of motor neurons is likely due to physiological and biological properties of these cells such as the increased amount of calcium buffering protein, the lack of direct monosynaptic corticospinal innervation, glutamatergic neurotransmission or nitric oxide metabolism.

1.4.3 Non-motor neuron pathology in ALS

In some cases of ALS, other CNS structures can be partially involved during the progression of the pathology. The extra motor neuron structures involved are the *sensory system*, *cerebellar pathways* and *substantia nigra*.

At autopsy pallor of the dorsal funiculi (dorsal root entry zone in the funiculus cuneatus and in the funiculus graciles) of the spinal cord which belong to the sensory system was observed in familial ALS cases. However, a degeneration of

the sensitive neurons was not present in ALS cases. Sporadically, it has been also observed that atrophy occurs in the posterior root ganglia, peripheral sensory nerve and thalamic gliosis.

Also frequent myelin pallor in the lateral and ventral white matter has been revealed in ALS patients, representing a compromised ascending spinocerebellar tract pathway during the progression of the disease. A subclinical Parkinsonism (no clear evidences of a Lewy body degeneration), developed from a degeneration of dopaminergic neuron in the substantia nigra, was present in some cases of ALS patients (Burrow and Blumbergs, 1992; Kato et al., 1993).

1.4.4 Non-CNS pathology in ALS

A direct consequence of the degeneration of the motor neuron is a skeletal muscle atrophy characterized by fibres clustering derived from serial denervation and reinnervation of the muscle. The phenomenon of sprouting is also evident as a tendency to restore the muscle innervation (Wohlfart, 1957).

Other tissues are involved during the progression of the disease, such as changes in collagen cross-linking or in the proportion of immature collagen, and rapid degeneration of the elastin component (Ono and Yamauchi, 1994).

1.5 MOLECULAR PATHOLOGY

The ALS pathology determines a series of abnormalities in the cytoplasm of motor neurons and glial cells which are represented by intracellular inclusion in the anterior horn cells and in other neuronal populations. Most of these inclusions are specific for ALS but some are shared with other neurodegenerative diseases, such as neurofibrillary tangles in motor neurons in patients with the ALS-Parkinson's disease dementia complex of GUAM.

The most specific features of the ALS neuropathology at the autopsy tissues analysis from patients are represented by:

- *Ubiquitin inclusion*
- *Bunina Bodies*
- *Hyaline conglomerate inclusions*
- *Golgi apparatus alterations*
- *Globules and spheroids*

1.5.1 Ubiquitinated Bodies Inclusions (UBI)

Tissues autopsy of ALS patients revealed skein-like inclusions or Lewy body-like accumulation of ubiquitinated substrates in the LMN. These inclusions occur specifically in the nervous system of ALS patients and are very infrequent in patients with other types of neurodegeneration. Furthermore, the motor

neurons spared in ALS (the oculomotor nuclei and Onuf's nucleus) did not usually show ubiquitin inclusion.

These deposits were evident in the normal appearing neurons and in morphologically altered neurons, demonstrating there is no direct correlation between the presence of the inclusion bodies and the degeneration event. Ubiquitin protein is a 76 aminoacid protein distributed in all cells of the organism. The main role of this protein is to tag abnormal peptides that subsequently will be eliminated by an extra-lysosomal alternative energy dependent pathway of degradation that involves several enzymes: ubiquitin proteasome pathway (Hershko et al., 1980). Ubiquitin proteasome system or ubiquitin-26S proteasome system is a barrel-shaped multi-protein complex that specifically is able to degrade unwanted and misfolded proteins into short polypeptides and amino acids in an ATP-driven reaction. Several enzymes in cascade regulate this process: E1 ubiquitin activating, E2 ubiquitin conjugating, and E3 ubiquitin ligase enzymes. Through these enzymes the ubiquitin monomers are attached sequentially to target proteins. The polyubiquitinated proteins are then recognized by the 26S proteasome, a large ATP-dependent multicatalytic protease, which removes the ubiquitin chain and degrades the proteins to short peptides. The selection and specific timing of polyubiquitination of the target proteins are conferred by different E3 ubiquitin ligases. As well as degrading misfolded proteins, the proteasome is involved in destruction of proteins that are regulated temporally (e.g. cell cycle regulators), or by environmental factors (e.g. cholesterol synthesis regulators and several transcription factors). Furthermore, ubiquitin is involved in cell stress responses and is also a heat shock protein involved in several insults to the cells (Bond and Schlesinger, 1985). The ubiquitin inclusions are also characterized by the

presence of the filaments and tubules of 15-25 nm in diameter (Lowe et al., 1988; Murayama et al., 1989; Murayama et al., 1990; Mizusawa et al., 1991; Schiffer et al., 1991) and smaller filaments of 10-15 nm in diameter. These inclusions do not show deposits of tau, α -synuclein and neurofilaments such as revealed in other neuropathology. UBIs are thought to result from the breakdown of abnormal proteins caused by oxidative stress, implicated in the pathogenesis of ALS (Alves-Rodrigues et al., 1998). This lesion characterized at least 80-100% of sporadic cases.

The ubiquitinated inclusions found in ALS have been classified as *skein-like* and *Lewy body-like* inclusions. Skein-like inclusions are a specific hallmark of ALS, whereas Lewy body-like inclusions are not so specific for ALS. Both the inclusions probably represent two different morphological stages of protein aggregations, from diffuse filamentous forms to dense and compact inclusions. In skein-like arrays, ubiquitin labelling is concentrated on abnormally formed 15-20 nm filaments and on neurofilament immunostaining localized on 10 nm filaments adjacent to or in continuity with the abnormal filaments (Migheli et al., 1994). Besides phosphorylated neurofilaments and ubiquitin, Lewy body-like inclusions also contain CDK5 kinase, a cyclin-dependent kinase (Nakamura et al., 1997). Moreover, in inclusion bodies found in the motor neurons and neuronal processes of familial and sporadic ALS, has been revealed dorf, a RING finger-type E3 ubiquitin ligase, (Hishikawa et al., 2003) and activated p38MAPK (Bendotti et al., 2004), an intracellular signalling kinase involved in stress stimulus and inflammatory events.

The tissue of ALS showed both skein-like inclusion and Lewy body-like ubiquitin inclusion, with a predominance of one type in some cases.

1.5.2 Bunina bodies (BBs)

BBs, which are cystatin C-containing inclusions, are found in the cell bodies of motor neurons in ALS (Okamoto et al., 1993; Sasaki and Maruyama, 1994). They are represented by small eosinophilic bodies composed by lysosomal material and they have been described in 30-50% of sporadic and familial ALS patients. However, they are now thought to be less specific for ALS than the ubiquitinated and neurofilamentous inclusions.

1.5.3 Hyaline conglomerate inclusions

These inclusions found in the spinal cord of ALS patients, are less specific for this neuropathology, as they have been also described in normal subjects and in other neurological diseases (Leigh et al., 1989; Sobue et al., 1990) although at a lower frequency. These inclusions are composed by aggregates of phosphorylated and non phosphorylated neurofilaments of 10-15 nm that are able to entrap other proteins and organelles (Hirano et al., 1967; Carpenter, 1968; Schochet et al., 1969; Kondo et al., 1986; Sasaki et al., 1988; Mizusawa et al., 1989). However, these aggregates were much less common than UBIs.

1.5.4 Fragmented Golgi apparatus (GA)

The GA is a crucial component of the cell, because it is involved in the transport and processing of polypeptides as well as in their targeting to different destinations, such as plasma membranes and lysosomes (Farquhar and Palade, 1981; Farquhar, 1985). Several studies revealed that the GA of the anterior horn cells is fragmented in sporadic (Kato et al., 1987; Gonatas et al., 1992; Matsumoto et al., 1996; Gonatas et al., 1998; Fujita et al., 1999; Fujita et al., 2002) and familial ALS patients (Fujita et al., 2000). This lesion of the GA consists of the dispersion of the normal network of the organelle into numerous small disconnected elements suggesting that the organelle is fragmented. Furthermore, fragmented GA, with an identical pattern to that observed in patients with ALS, was revealed in asymptomatic transgenic mice expressing G93A mutation before the onset of paralysis, suggesting that this alteration is an early and specific target of the pathological process (Mourelatos et al., 1996). These studies suggested that fragmentation of a structure with such crucial roles in the cellular handling of proteins was likely to be associated with significant impairment of function, revealing another possible early target for the motor neuron degeneration.

1.5.5 Globules and spheroids

Another important alteration revealed in the autopsy of ALS patients is the axonal swellings in the anterior horn. This is due to disoriented conglomeration of phosphorylated neurofilaments called spheroids and globules. Spheroids tend

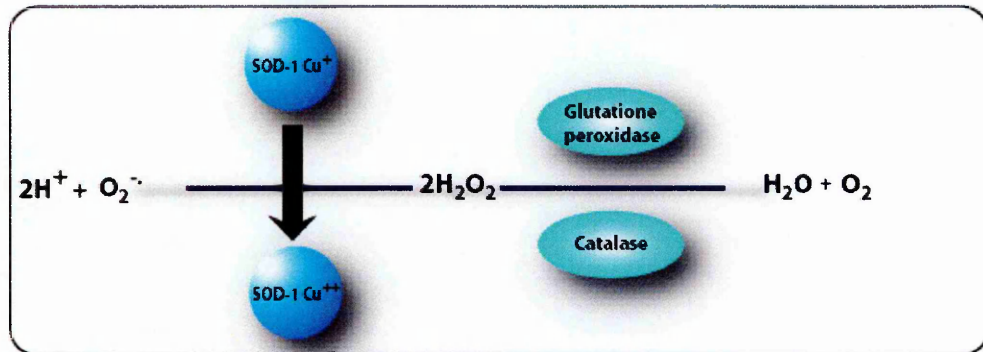
to be in proximal axons, while globules are present much more distal in the ventral horn (Sobue et al., 1990). Some spheroids may be also in dendrites (Sasaki et al., 1988). A deregulated anterograde transport of neurofilaments was hypothesized with this alteration.

1.6 GENETICS OF ALS

The studies of Charcot on 20 ALS patients in the 1860s suggested that ALS pathology was not a hereditary disease. Subsequently, this view was challenged by other authors demonstrating an inheritance pattern of the disease (Kurland and Mulder, 1955; Cudkowicz et al., 1997). Other studies confirmed that the familial form of ALS (FALS) can be inherited as an autosomal trait (9-10%). The clinical features of sporadic and familial ALS are not distinguishable although FALS shows an earlier onset which is more pronounced at the lower extremities (Mulder et al., 1986).

An important contribution on the pathogenesis of ALS was made by Rosen and colleagues. In 1993 this group reported that some FALS families are genetically linked to chromosome 21q22.1-22.2 and the gene involved was the SOD1 (Rosen et al., 1993). SOD1 is a cytoplasmic homodimeric enzyme ubiquitously distributed in eukaryotic cells which is coded by five exons and each subunit consists of 153 amino acids. In the nervous system it represents 2% of the proteins. Its functions consist in the catalysis of free radical superoxide, generated mainly from the oxidative phosphorylation in the mitochondria, to hydrogen peroxide and oxygen mediated by copper (Fig 1.0).

Fig 1.0: SOD1 chemistry



It was initially proposed that the toxicity of mutated SOD1 is associated with the loss of superoxide dismutase activity. However, most mutated SOD1 forms appear to fully retain their enzymatic property. The creation of transgenic mice expressing SOD1 with some of the mutations found in the human patients, which develop a motor syndrome similar to human ALS, shows that catalytic activity is unchanged or elevated (Gurney et al., 1994; Ripps et al., 1995). Furthermore, SOD1 knockout mice do not develop spontaneous motor neuron disease (Reaume et al., 1996; Wong et al., 1995; Bruijn et al., 1997b). The conclusion is that SOD1 mutants acquire one or more toxic properties, irrespective of the amount of superoxide dismutase activity that each of them retains.

So far more than one hundred different mutations have been revealed in the Cu/Zn-SOD-1 gene of FALS patients. Almost all the mutations are dominant missense mutations (an aminoacid substitution), except for SOD1D90A

mutation that has a recessive trait. However, the variability of the gene mutation does not determine a distinguishable clinical phenotype.

Recently another 2 genes have been shown to be involved in recessive inheritance and juvenile ALS form. One genetic alteration was revealed in 7 Tunisian families with a mutation locus localized in 15q15.22, but the gene has not been identified so far. The second gene is mapped to chromosome 2q33 that encodes a 184 Kda protein derived from 34 exons that span 80Kb and showed deletion mutations (Yang et al., 2001). This gene, called ALS2 or *Alsin*, is widely expressed and contains an amino acid sequence in the amino-terminal that belongs to guanine exchange factors (GEFs) that are involved in the exchange from the GDP bound state to the GTP state in a small G protein. The carboxy-terminal contains a domain characteristic of the Rho G-protein family, which is able to modulate dynamic actin assembly.

Other gene mutations that are rarely causative in ALS include deletions or insertions in the neurofilament genes. In fact, alterations in the NF heavy subunits genes in the lysine-serine-proline (KSP) motifs were identified in a small percentage of ALS patients (1% of 1300 sporadic ALS patients) (Al-Chalabi et al., 1999). Other gene alterations were revealed such as mutations in mitochondrial DNA encoded subunit I of cytochrome C oxidase (Borthwick et al., 1999) or abnormal SMN1 (Survival Motor Neuron 1) gene copy number (Corcia et al., 2002) and NAIP (Neuronal Apoptosis Inhibitor Protein) mutations (Jackson et al., 1996). These latter two genes are mainly involved in the survival of motor neurons. Furthermore, variant mRNA transcripts for the astroglial glutamate transporter excitatory amino acid transporter (EAAT2 in human or GLT-1 in mouse) were showed in the central nervous system of sporadic ALS (SALS) patients (Lin et al., 1998). Other loci mapped to

chromosome 9q34 were identified in a juvenile form of ALS characterized by a very slow progression of distal limb atrophy and motor neuron loss that does not reduce the life span (Chance et al., 1998). The gene involved in these mutations, *Senataxin* gene, encodes for a protein that contain a DNA/RNA helicase domain with strong homology to genes such as human RENT1 and IGHMBP2, which encode proteins involved in RNA processing (Chen et al., 2004). A debrisoquine hydroxylase gene polymorphism was also described in patients with ALS (Siddons et al., 1996). This encodes for a cytochrome P450 monooxygenase protein involved in drug metabolism and associated with a poor metabolism phenotype. Furthermore, a novel missense mutation in the vesicle-associated membrane protein/synaptobrevin-associated membrane protein B (VAPB) gene was reported in ALS patients (Nishimura et al., 2004). Vesicle-associated proteins are intracellular membrane proteins that are associated with microtubules and have a function in membrane transport (Nishimura et al., 2004).

1.7 ANIMAL MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

The discovery of the SOD1 mutant gene by Rosen *et al.* (Rosen et al., 1993) as the cause of a subgroup of FALS has led to the generation of transgenic mice carrying the human SOD1 gene with different mutations. These mice develop a phenotype similar in many aspects to human ALS pathology. Different lines of transgenic mice overexpressing human mutant SOD1 (SOD1G93A, SOD1G37R and SOD1G85R) or mouse mutant SOD1 (SOD1G86R) have been produced. More recently rat models with SOD1G93A and SOD1H46R have also been developed (Nagai et al., 2001). SOD1G93A mice are the most used animal models as they have been commercially available for a long time.

All these animals develop a motor syndrome characterized by selective motor neuron death. They show normal motor functions in the early phase of their life but then develop a progressive weakness, especially starting in the hind limbs, which eventually results in paralysis and death. The age of onset, the duration and several pathological features of this motor dysfunction show some differences in the transgenic animals' strains. However, all these transgenic mice and rats provide an excellent and suitable *in vivo* model resembling human ALS.

Mice expressing SOD1G85R develop a late onset but very aggressive pathology (2 weeks from the first symptoms to the death), even with a low expression of the transgene. Motor neurons and astrocytes present inclusions immunopositive for ubiquitin and SOD1 (Bruijn et al., 1997b).

Transgenic mice expressing low level of SOD1G37R mutant shows a motor disease restricted to LMN, whereas higher copy number causes more severe

abnormalities and affect a variety of other neuronal populations. The most obvious cellular abnormality is the presence in axons and dendrites of membrane-bounded vacuoles, which appear to be derived from degenerating mitochondria (Wong et al., 1995).

Transgenic mice that express mutant SOD1G93A develop motor system disease mainly affecting motor neurons. Ultrastructural and microscopical analysis reveals that the earliest pathological feature in these mice is vacuolization of large neurons of the anterior horns of the spinal cord (Bendotti et al., 2001a). At the end stage, motor neuronal depletion is evident and hyaline filamentous inclusions immunopositive for ubiquitin and neurofilaments are present in some of the surviving neurons (Gurney et al., 1994; Migheli et al., 1994).

It is noteworthy that transgenic mice overexpressing the wild type form of human SOD1 (SOD1WT) do not develop any motor symptoms or altered phenotype. However, some neurodegenerative changes consisting of swelling and vacuolization of mitochondria, axonal degeneration of some long fiber tracts and a moderate loss of spinal motor neurons at two years of age, were reported (Jaarsma et al., 2000). Thus, SOD1WT transgenic mice have demonstrated that mutations on SOD1 gene are necessary to induce an evident motor disease. Therefore, transgenic technology has directly clarified that SOD1 mutants cause pathology because of gain of function and not because of the loss of SOD1 catalytic properties. Moreover, expression of mutant forms of SOD1 restricted to astrocytes or neurons is not sufficient to cause motor neuron degeneration in transgenic mice (Gong et al., 2000; Pramatarova et al., 2001; Lino et al., 2002). These reports suggest that simultaneous alterations in both neuronal and astroglial cells are necessary to induce the pathology in ALS mice.

More recently, the transgenic technology has also permitted the creation of transgenic rats expressing the G93A mutant of SOD1. Motor neuron disease in these animals depends on high levels of mutant SOD1 expression. Disease onset in SOD1G93A rats is quite early and disease progression is very rapid (11 days to reach the end stage). Pathological abnormalities include vacuoles initially in the lumbar spinal cord and subsequently in more cervical areas, with inclusion bodies that stain for SOD1, Hsp70, neurofilaments and ubiquitin. Vacuolization and gliosis are evident before clinical onset and before motor neuron death in the spinal cord and brainstem (Howland et al., 2002).

Furthermore, mice carrying naturally occurring autosomal recessive mutations on unidentified genes provide animal models with motor system impairment. *PMN mice* show a progressive motor neuronopathy characterised by paralysis of the limbs, neurogenic atrophy of muscles, axonal degeneration but with relative sparing of motor neuron cell body. The PMN locus was identified on chromosome 13, but the gene involved is not known (Brunialti et al., 1995).

The *wobbler mice* represent another model of motor neuron disease. They show a progressive forelimb weakness associated with proximal axonal degeneration and vacuolar changes within the anterior horn cells of the spinal cord with a little involvement of the brain. In this case, the gene responsible for this syndrome has been mapped on chromosome 11 (Kaupmann et al., 1992), and recently has been identified as a missense mutation in Vps54 gene associated with vacuolar vesicular sorting factors involved in the vesicular trafficking (Schmitt-John et al., 2005).

Mice showing neuromuscular degeneration, with autosomal recessive mutation localized on the gene coding for the ATPase/DNA helicase were described

(Cook et al., 1995; Cox et al., 1998). Called *nmd mice*, they present rapidly progressive hindlimb weakness and motor neuron cell body degeneration.

Another spontaneous animal model of motor dysfunction is represented by *mnd mice*, considered in the past a model of ALS. These mice develop a late onset motor neuron degeneration characterized by progressive deterioration of motor function which is more severe in the lumbosacral than in the other regions, as well as variable pathology in the lower cranial nerves (Messer et al., 1987). However, the number of choline acetyltransferase (ChAT) immunopositive lumbar motor neurons is not different from normal mice (Mennini et al., 2002). The MND mice carry a spontaneous homozygous mutation in the coding region of the gene *Cln8* belonging to the family of neuronal ceroid lipofuscinose-related genes (CLNs) (Ranta et al., 1999). Thus, the presence of abnormal autofluorescent cytoplasmic inclusions rich in lipofuscin found in neurons, but also in many other somatic organs, makes these animals a useful model for human neuronal ceroid lipofuscinosis rather than for ALS (Bronson et al., 1993).

Recently, Ahmad-Annuar and colleagues have described another mutant mouse model of motor neuron disease, the legs-at-odd-angles (*Loa*) mutant. In these mice mutations in the cytoplasmic dynein heavy chain gene (*Dnchc1*) cause motor neuron degeneration. Mice exhibiting the *Loa* phenotype suffer progressive loss of locomotor function and homozygous animals have neuronal inclusion bodies that are positive for SOD1, CDK5, neurofilament and ubiquitin proteins (Hafezparast et al., 2003). As this phenotype reproduces some aspects of human motor neuron degeneration disorders, it is possible that dynein may be a causative gene or susceptibility factor in human motor neuron disease.

However, to date, no association between familial motor neuron disease and the genotypes presented by Loa mice has been found.

All these mice strains offer several advantages in the study of early phenomena occurring in a motor neuron disorder, involving a progressive and naturally occurring impairment of motor system. However, many features are quite dissimilar from human ALS pathology. Moreover, it is possible that the genetic alterations affecting these mice and the associated biochemical defects do not contribute to the human disease. Thus, these models are not particularly reliable for testing therapeutic intervention or uncovering disease mechanisms, although the study of the pathology occurring in these mouse models may contribute to the discovery of still unknown risk factors for human ALS.

1.8 THE THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS

No cure for ALS patients so far is available, and the only drug approved in the 1996 by the Food and Drug Administration is riluzole (Miller et al., 1996), although it produces only a modest effect in prolonging the survival of patients and mouse models by a few months (Gurney et al., 1998; Riviere et al., 1998). Different mechanisms were proposed by experiments in vivo and in vitro for this drug, such as inhibition of the release of glutamate due to the inactivation of voltage-dependent Na^+ channels on nerve terminals, as well activation of a G-protein dependent signal transduction process (Doble, 1996). Furthermore, it was demonstrated that riluzole can block some of the postsynaptic effects of glutamate by non-competitive inhibition of NMDA and AMPA receptors (Doble, 1996; Albo et al., 2004). In addition, it has been demonstrated in ALS patients using proton magnetic resonance spectroscopy that riluzole reduces the amount of motor neuron loss during the progression of the disease (Kalra et al., 1998).

Other pharmacological approaches were attempted to block or ameliorate the motor neuron loss, among them, the use of specific neurotrophic factors such as BDNF (Brain Derived Neurotrophic Factor) and IGF-1 (Insulin growth factor). Several studies have shown a neuroprotective effect in animal models for these trophic factors, evidenced by preserving the morphology of motoneurons, decreasing gliosis and increasing the lifespan of SOD1 transgenic mice. For IGF-1 two clinical trials were carried out in North America that have shown an opposing effect; however only one resulted in an improved life span (Lai et al., 1997). Furthermore, a European trial have confirmed the lack of efficacy of this

factor (Borasio et al., 1998). Also for BDNF, different trials were carried out in America and in North America utilizing subcutaneous and intrathecal administration. However no amelioration of the progression of the disease was detected (Clinical Trial Summary, Motor Neuron Disease Association Web site). Vascular endothelial growth factor (VEGF) also has a neuroprotective effect on motoneurons and increases the lifespan of SOD1 mutant mice and rats (Azzouz et al., 2004; Storkebaum et al., 2005). Furthermore, VEGF has been shown to be a modifier of motor neuron degeneration in ALS patients (Lambrechts et al., 2003).

Recently, it has been demonstrated that the method of delivery of a treatment is important. An interesting example is treatment with IGF-1 or VEGF retrogradely transported in motoneurons through viral vectors that were injected into muscles (Kaspar et al. 2003; McGeer and McGeer 2005). This produced one of the best results obtained in mice models, even when delivered at the time of clear disease symptoms.

Other drugs were proposed and tested such as the tetracycline antibiotic minocycline, whose role seems to be to inhibit p38 mitogen activated protein, cytochrome C released from the mitochondria, inducible nitric oxide synthetase (iNOS), caspase 3 and 1 (Tikka et al., 2001; Zhu et al., 2002). This drug has shown promising effect on culture cells and in animal models of different pathology such as Parkinson's disease, stroke, trauma, and Huntington Disease (Yrjanheikki et al., 1998; Yrjanheikki et al., 1999; Chen et al., 2000; Wu et al., 2002). In addition, a positive effect has been detected in transgenic mouse model of ALS (Kriz et al., 2002; Zhu et al., 2002). A trial has been opened for this drug that has reached the phase III.

Interesting neuroprotective effects was shown in transgenic mouse model for creatine, which acts on reduced energy metabolism and/or mitochondrial dysfunction (Klivenyi et al., 1999) or cyclooxygenase 2 inhibitors (Klivenyi et al., 2004), and has an anti-inflammatory effect. However, no effect was demonstrated using these drugs in ALS patients (Shefner et al., 2004).

Other substances able to reduce the glutamate excitotoxicity were considered such as ceftriaxone which, among other effects, increases the expression of GLT1 and moderately prolongs the survival of SOD1G93A mice (Rothstein et al., 2005). On this basis, a long-term double-blind clinical trial was proposed, despite the negative results obtained from eight clinical trials with short-term (five to eight weeks) ceftriaxone treatment (Beghi et al., 2005).

However, neither gabapentine nor topiramate, other antiglutamate drugs, exert beneficial effects in preclinical and clinical trials (Gordon, 2005). Instead, other anti-excitotoxic agents able to inhibit AMPA receptors have shown beneficial effects in mouse models (Canton et al., 2001; Van Damme et al., 2003; Tortarolo et al., 2006), and a clinical trial with talampanel has recently been proposed (Andersen et al., 2005).

A promising approach might be a simultaneous treatment using molecules that act on different targets. Indeed, animal studies have shown that combination therapies often have synergistic effects. For example, treatment with minocycline produces an additional beneficial effect in transgenic mice when administered with creatine (Zhang et al., 2003).

Promising effects have also been shown using cellular therapy by stem cells (Silani et al., 2002). A significant effect in prolonging the survival of SOD1G93A mice was reported in a study using human umbilical-cord blood cells injected intravenously (Garbuzova-Davis et al., 2003). Human neural

progenitor cells modified with GDNF (Klein et al., 2005), or Sertoli cells (Hemendinger et al., 2005), injected into the spinal cord of SOD1G93A rats induced a significant neuroprotection in the proximity of the site of implantation.

Furthermore, some studies have suggested that a high energy diet has a compensating effect on the energetic imbalance of the transgenic mouse model (Dupuis et al., 2004).

1.9 GLUTAMATE RECEPTORS

The transmission of glutamate stimulus is regulated by four different receptors that can be subdivided in ionotropic and non ionotropic glutamate receptors. These can be furthermore subdivided such as listed:

- **IONOTROPIC RECEPTORS**

- AMPA

- Kainate

- NMDA

- **NON IONOTROPIC RECEPTORS**

- Metabotropic receptors

The fundamental difference between these two kinds of receptors is that ionotropic receptors are ions channels, whereas non-ionotropic receptors act through an intracellular signaling cascade that can influence ions channels.

1.9.1 IONOTROPIC RECEPTORS

Ionotropic receptors are characterized by a multimeric structure composed of different subunits. They are divided based on pharmacological and structural similarities into NMDA (N-methyl-D-aspartate), AMPA (alpha-amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid) and Kainate receptors. They are structurally characterized by four transmembrane domains with an extracellular N-terminal and intracellular C-terminal. The second domain forms the channel pore through a re-entrant loop. A loop between the III and IV domains forms a binding site for the ligand (Standley and Baudry, 2000).

1.9.1.1 NMDA RECEPTORS

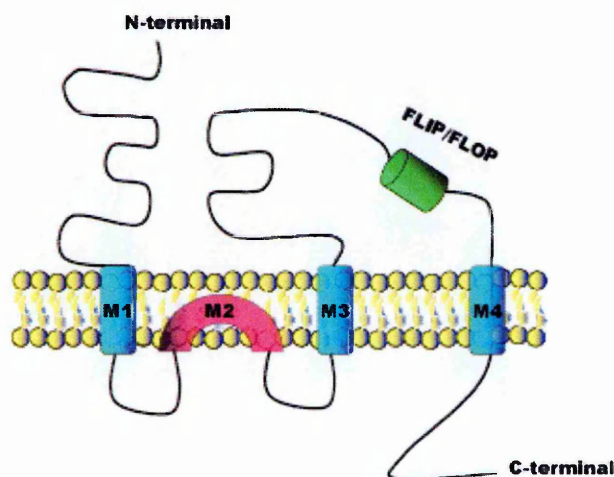
They are considered the most important glutamate receptor because they are able to regulate the calcium permeability in cells in physiological conditions. The ion influx is regulated by magnesium that is released in a voltage-dependent manner, and by a co-agonist glycine. Furthermore the activity is regulated by modulatory sites for polyamines, zinc ion, reducing agents and protons (Heath and Shaw, 2002). These receptors can be composed of different subunits: NR1, represented by 8 isoforms (Zukin and Bennett, 1995) and NR2, present in 4 isoforms, NR1 is always present in functional NMDA receptors whereas NR2 determines the pharmacological and physiological properties of the receptor (Meguro et al., 1992). An ubiquitous distribution of NR1 was demonstrated, whereas NR2 is much more localized in specific sites. A third subunit has been found recently, NR3, whose role so far has not been characterized (Das et al., 1998).

1.9.1.2 AMPA RECEPTORS:

Originally identified based on its activation by non selective agonists, it was subsequently demonstrated to be activated selectively by a more specific agonist, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). Structurally it is composed of 4 different subunit proteins (GluR1-2-3-4) in a homomeric or heteromeric composition derived from different genes.

Current models for the topology of GluRs suggest that they comprise an extracellular amino terminal with consensus glycosylation sites, four hydrophobic membrane associated domains of which 1, 3 and 4 are transmembrane spanning while the second forms a reentrant loop, and an intracellular carboxyl terminal region with consensus phosphorylation sites (Fig.1.1)

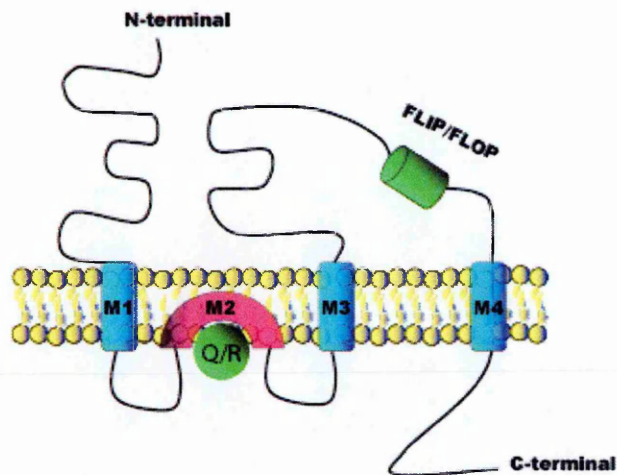
Fig 1.1: GluR receptor structure



A functional AMPA receptor differs in subunit composition depending on both nervous system region and neuronal subtype. AMPA receptor composition is

not rigid but it can change and is dynamically influenced by the environmental conditions (Luscher et al., 2000; Zhou et al., 2001). The most important subunits able to regulate calcium entry are the GluR2 subunits, that have an editing site in the second transmembrane domain Q/R site which is able to change a glutamine with an arginine in an edited state (Michaelis, 1998; Dingledine et al., 1999) (Fig.1.2).

Fig 1.2: Q/R editing site



This site is developmentally regulated and completely edited with the arginine in the postnatal mammal. The presence of GluR2 in the composition of the AMPA receptors determines through a positively charged arginine a block on calcium entry. Other splicing and editing that determine different aminoacidic sequences characterize the AMPA receptor subunits. A FLIP/FLOP isoform variant defined by a 30 amino acids cassette and an editing site R/G (arginine/glycine) located in the loop between the third and fourth transmembrane domain determines the desensitized state of the receptor once activated. An increased capacity of desensitization of AMPA receptors with a

FLOP isoform rather with a FLIP isoform has been demonstrated (Myers et al., 1998). Furthermore, a glycine instead of the arginine in the R/G determines an increased return to the activated state of the AMPA receptors. Hence, an altered regulation at these sites is able to result in a dysfunction in the homeostasis of ion influx, with deleterious consequences for the neuronal cell involved.

A much fast kinetics characterizes these receptors in comparison to NMDA receptors when activated in synaptic transmission.

1.9.1.3 KAINATE RECEPTORS:

This kind of ionic receptor is distinguished from NMDA and AMPA receptors by a selective activation by the kainate agonist. Structurally they have a similar amino acid sequence to AMPA receptor subunits, with 4 transmembrane domains with a N-extracellular and a C-intracytoplasmic domain. However, they have a particular N-terminal sequence that leads to specific binding by the kainate agonist. A functional receptor is characterized by heteromeric assembly of 5 different subunits: GluR 5-6-7 and KA 1-2, with the particular subunit composition determining the receptor's pharmacological and functional properties.

Also in this case, an editing or a splicing activity gives rise to kainate receptors having different ion conductance and desensitization.

1.9.2 NON-IONOTROPIC RECEPTORS

A completely different structure and function is associated with this type of glutamate receptor in contrast to the ionotropic receptors. They belong to the G-protein coupled receptor family with eight transmembrane domains.

1.9.2.1 METABOTROPIC RECEPTORS:

Each receptor can be functionally constituted by eight different subunits (mGLUR1-8) which are grouped in three major families: Group I represented by mGluR1 and mGluR5 having a transduction mechanism associated with postsynaptic inositol phosphate metabolism. In contrast mGluR2, 3 and mGluR4, 6, 7, 8 have been associated respectively with the Group II and Group III. Both are coupled to an inhibitory cAMP cascade in many expression systems. These receptors were located frequently at the presynaptic membrane, with the role of modulating the function of glutamate at this site (Heath and Shaw, 2002).

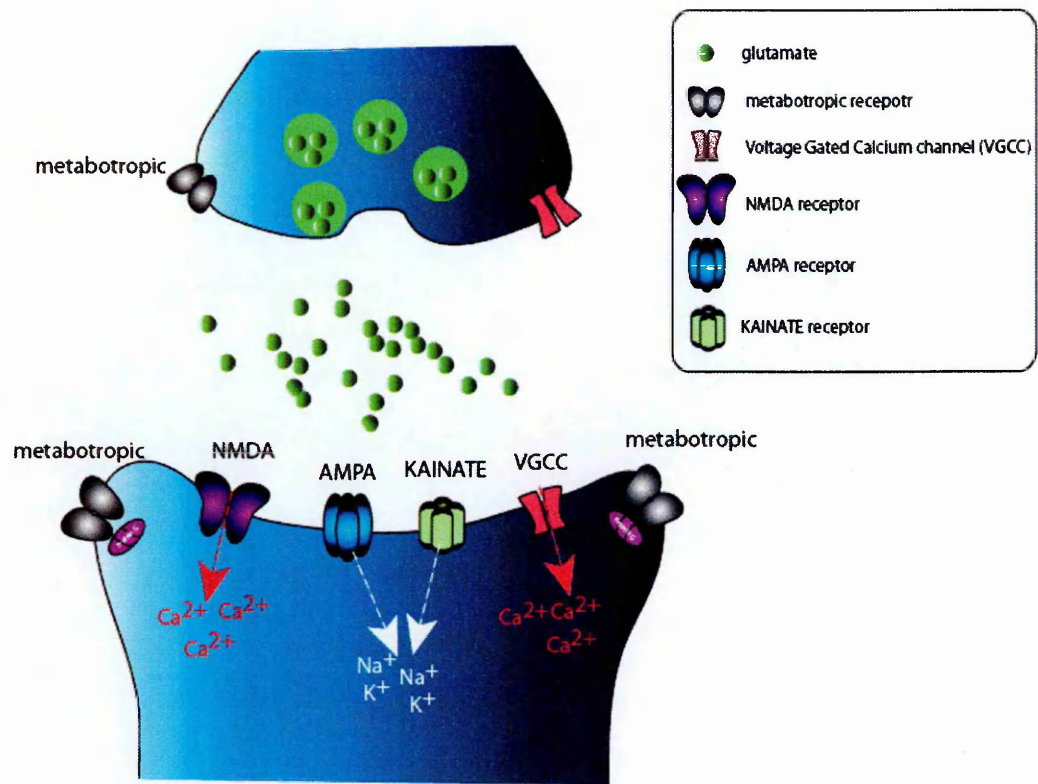


Fig 1.3: Ion Channels

Ion channels are pore-forming proteins that help to establish and control the small voltage gradient across the plasma membrane of all living cells by allowing the flow of ions down their electrochemical gradient.

L-Glutamate is the major excitatory neurotransmitter in the mammalian CNS, acting through both ligand gated ion channels (ionotropic receptors) such as NMDA, AMPA and Kainate receptors and G-protein coupled (metabotropic) receptors. Activation of these receptors is responsible for basal excitatory synaptic transmission and many forms of synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD). Noteworthy for the excitotoxicity events are the ligand gated calcium channels such as NMDA and

AMPA. Others receptors are able to regulate the calcium entry such as voltage gated calcium channels (VGCC).

1.10 REGULATORY MECHANISMS OF CALCIUM ENTRY BY AMPA RECEPTORS

The calcium entry through AMPA receptors is regulated by different molecular features of their own subunits, such as: the presence of edited form of the GluR2 subunit, channel desensitization, and receptor density on the cell's surface.

1.10.1 EDITED FORM OF THE GLUR2 SUBUNIT

A post-transcriptional change through the editing of adenine (A) to inosine (I) in the receptor RNA leads to an editing changes CAG into CIG (Sommer et al., 1991; Kim et al., 1994; Melcher et al., 1996), hence a substitution of the Glutamine (G) with the Arginine (R) at the Q/R site located in the second trans-membrane domain of the AMPA receptor subunit. All of the AMPA receptor subunit genes encode glutamine at this Q/R site; however GluR2 alone is featured for the presence of an R substitution at this level determining a block of the calcium entry. This editing has an efficiency of 100% in the mammalian neurons from an embryonic stage throughout life (Burnashev et al., 1992; Nutt and Kamboj, 1994). The enzyme involved in the editing of GluR2 is adenosine deaminase acting on RNA type 2 (ADAR2) (Melcher et al., 1996; Higuchi et al., 2000). Hence, the presence at least of one edited GluR2 subunits determines a block of calcium permeability, whereas the presence of the unedited subunits determine an increase influx of this ion leading to excitotoxic events (Hollmann et al., 1991; Hume et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1995; Kuner et al., 2001).

1.10.2 CHANNEL DESENSITIZATION

Another mechanism of modulation that enables the regulation the homeostasis of calcium is the kinetics of desensitization of the receptor, which determines the resting state of the AMPA receptors. An aminoacidic substitution (arginine R/glycine G) in the loop between the third and fourth transmembrane domain at the R/G site and a cassette of 38 aminoacids that exist in two different forms (FLIP-FLOP) regulates the potential desensitization of these receptors. It was demonstrated that the edited glycine substitution in the R/G site has a faster recovery from desensitization state than the unedited arginine isoform (Lomeli et al., 1994). Two enzymes called ADAR1 and ADAR2 catalyze the editing for GluR 2, 3 and 4 (Melcher et al., 1996; Higuchi et al., 2000). Furthermore, it has been demonstrated that the FLIP form desensitizes more slowly than the FLOP isoform (Sommer et al., 1990; Lambolez et al., 1996), leading to an increased calcium entry.

1.10.3 RECEPTOR TRAFFICKING AT THE POSTSYNAPTIC SPACE

AMPA receptors change dynamically and their trafficking is regulated by peculiar proteins. An altered trafficking of the AMPA receptor subunits to the postsynaptic membrane could lead to an uncontrolled influx of ions in the cytoplasm, breaking the mechanism that regulates this homeostasis.

Recently several proteins were discovered to regulate trafficking of glutamate receptors between the cytoplasm and the membrane. The proteins involved in the trafficking of the GluR subunits interact with a specific amino acid

sequence localized in the intracellular cytoplasmic tails of the glutamate receptors and they themselves contain in their sequence a PDZ domain (Post-synaptic density-95/Discs large/Zona occludens-1) (Doyle et al., 1996). PDZ is a modular domain for protein–protein interaction also known as Discs-Large homology Repeats (DHR) of approximately 100 amino acid domains originally identified on repeated regions of the synaptic protein PSD-95 in the brain, in the *Drosophila* septate junction protein Discs-Large, and in the epithelial tight junction protein ZO1 (Woods and Bryant, 1991; Cho et al., 1992; Willott et al., 1993). PDZ sequence is considered a modular protein-protein interaction motif that serves to localize proteins to specific subcellular sites.

PDZ domain-containing proteins have been shown to mediate protein-protein interactions during receptor and ion channel clustering and to recruit kinases and phosphatases to their membrane-associated substrates. Certain PDZ domains have also been shown to homooligomerize, thereby contributing to the formation of multiprotein complexes at specific subcellular sites (Brenman et al., 1996).

A splice cassette dictating long and short forms of the subunit was detected in the C- terminal of both GluR2 and GluR4 subunits (Gallo et al., 1992; Kohler et al., 1994). The predominant form of GluR2 is the short version (GluR2short) with less than 10% of the GluR2 transcripts in mouse brain encoding GluR2long (Kohler et al., 1994). GluR4short is expressed preferentially in granule neurons of the cerebellum and Bergmann glial cell bodies. The GluR4long form is restricted to Bergmann glial cells in the cerebellum, but is also expressed neuronally in the forebrain, mostly in interneurons (Gallo et al., 1992). Long variants have C-terminal domains that contain 68-81 amino acids while short isoforms contain 50 residues. Both long and short variants of each

subunit are identical up to a lysine residue located 14 amino acids downstream of the last membrane domain (M4). In the short forms of GluR2 and GluR4 the entire C-terminal is encoded on the same exon that encodes for M4 whereas in the long splice variants the remaining C-terminal is encoded by the next exon which contains the 3% untranslated region of short isoform. To date, no short form of GluR1 or long version of GluR3 has yet been detected (Gallo et al., 1992; Hollmann and Heinemann, 1994; Kohler et al., 1994). The short (but not the long) forms of the AMPA receptor subunits contain a T:S X V-like motif (also called PDZ-binding motif) at the extreme C-terminal. This motif interacts with proteins containing a PDZ domain and the interaction is thought to localise proteins to specific subcellular regions. It has been proposed that the PDZ domain provides an important mechanism for clustering ion channels and receptors to the plasma membrane and for directing kinases and phosphatases toward their substrates.

Differences were revealed in the C-terminal domains of the GluR subunits. GluR1, 4 and a splicing form of the GluR2long have longer cytoplasmic tails that are highly homologous (Kohler et al., 1994; Kolleker et al., 2003). Whereas, GluR2short, 3 and a splicing isoform of the GluR4short have shorter cytoplasmic homologous tails (Gallo et al., 1992; Kawahara et al., 2004a). Each subunit interacts with specific trafficking proteins that regulate the clustering in the membrane and in the subcellular compartment. The proteins involved in this trafficking can be subdivided into the **group-1 PDZ proteins** such as synapse associated protein 97 (SAP-97) that interact with the GluR1 subunits (Leonard et al., 1998), whereas GluR2-3-4 interact with the **group-2 PDZ proteins** represented by protein interacting with C-kinase 1 (PICK-1) (Dev et al., 1999), glutamate receptor interacting protein type 1 (GRIP1) and type 2 (GRIP2, also

know as AMPA receptors binding proteins; ABP). Another important **no PDZ protein** involved in the clustering of the AMPA receptor subunits into the membrane is NSF (N-ethylmaleimide sensitive fusion protein). Different features can be associated to these proteins and will be mentioned later.

The surface expression of the AMPA receptors cycle in a constitutive mode in and out of the plasma membrane (Luscher et al., 1999; Malinow et al., 2000; Carroll et al., 2001). AMPA receptor internalization from the cell surface occurs via a clathrin-mediated dynamin-dependent pathway and is stimulated by a variety of factors that induce synaptic depression (Carroll et al., 1999; Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Man et al., 2000; Wang and Linden, 2000). Furthermore, recent studies of AMPA endocytosis suggest different pathways of internalization of the AMPA receptors sorting to an endosomal compartment ready to be recycled to the surface or to a lysosomal compartment where they may be degraded (Ehlers, 2000; Lin et al., 2000). This supports the idea that there are two distinct pools of synaptic AMPA receptors, one that can be regulated by activity and one that serves a maintenance function (Carroll et al., 1999; Daw et al., 2000; Malinow et al., 2000; Carroll et al., 2001; Shi et al., 2001)d

1.10.4 PHOSPHORYLATION STATE OF THE AMPA RECEPTORS

A control of the regulated equilibrium between the cytosol and the membrane is associated also with the direct phosphorylation at multiple sites in the C-terminal tails of the AMPA receptor subunits. The phosphorylation of the AMPA receptor subunits can also modify the electrophysiological and

biochemical (synthesis and subunit composition) properties of the AMPA receptor subunits.

Recent studies have shown that the AMPA receptor subunits can be phosphorylated on their subunits GluR1, GluR2, and GluR4. The amino acids phosphorylated are serine, threonine, or tyrosine on the intracellular C-terminal domain. Several protein kinases, such as protein kinase A (PKA), protein kinase C (PKC), Ca^{2+} /calmodulin-dependent protein kinase II (CAMKII), and tyrosine kinases are involved in the site-specific regulation of the AMPA receptor phosphorylation.

Using site directed mutagenesis and phosphopeptide mapping analysis, two major phosphorylation sites on the serine 831 and serine 845 residues of the GluR1 C-terminus have been identified (Fig 1.3). These sites are specifically phosphorylated respectively by PKC and PKA, and it has been demonstrated that the latter is able to augment the AMPA receptor current (Roche et al., 1996). Others studies have demonstrated that serine 831 can also be phosphorylated by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) (Mammen et al., 1997; Strack et al., 1997; Vinade and Dosemeci, 2000), potentiating the GluR1 current (Lledo et al., 1995; Lisman and Zhabotinsky, 2001; Poncer et al., 2002). Furthermore, there is evidence that the phosphorylated forms of GluR1 facilitate the incorporation of AMPA receptors into synapses (Esteban et al., 2003).

<p>GluR1: EFCYKSRSESKRMKGFCCLIPQQSINEAIRTSTLPRNSGAGASGGGGSGENGRVVSQDFPKSMQSIPCMSHSSGMPLGATGL</p> <p>Ser831 Ser845</p>
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Fig 1.4: Amino acid sequence in the GluR1 C terminus
Phosphorylation sites on the Ser831 and 845 residues of the GluR1 subunit

Another subunit that also contains potential sites for protein phosphorylation is GluR2. Experiments of single site mutagenesis have demonstrated that the phosphorylation occurs on serines 863 and 880 of the C-terminus (Fig 1.4) (Matsuda et al., 1999; McDonald et al., 2001). The latter has been demonstrated to be phosphorylated by PKC (McDonald et al., 2001). In addition, GluR2 C terminus has multiple tyrosine residues underlying phosphorylation as tyrosine 876, a last tyrosine residue within the C terminal PDZ ligand domain which is phosphorylated by nonreceptor Src family tyrosine kinases. Interesting, the binding domain of some trafficking proteins such as ABP, GRIP and PICK1, overlaps with the phosphorylation sites on tyrosine 876 and serine 880. The phosphorylation status on these sites affects the association of the AMPA receptor subunits with the interacting proteins, thereby modifying trafficking and distribution of the receptor. Indeed, a disrupted interaction of GluR2 with GRIP leading to reduced surface expression has been revealed after phosphorylation of Glur2 (Chung et al., 2000; Hirai, 2001).

GluR2: EFCYKSRAEAKRMKVAKNPQNINPSSSQNSQNFATYKEGYNVYGIESVKI Ser863 Tyr876 Ser880
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Fig 1.5: Aminoacid sequence in the GluR2 C terminus
Phosphorylation sites on the Ser863, Ser880 and Tyr876 residues of the GluR2 subunit

In GluR4 the phosphorylation site was identified on serine 842 within the C terminal domain (Carvalho et al., 1999). Several kinases are able to phosphorylate in vitro serine 842, including PKA, PKC and CAMKII. Furthermore, another consensus site on threonine 830 was identified as a phosphorylation site for PKC (Pearson and Kemp, 1991) (Fig 1.5). PKA phosphorylation of GluR4 lead AMPA receptor subunits into synapses (Esteban et al., 2003)

GluR4: EFCYKSRAEAKRMKLTFSEAIRNKARLSITGSGVGENGRVLYPDCPKAVHTGTAIRQSSGLAVIASDLP Thr830 Ser842
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Fig 1.6: Aminoacid sequence in the GluR4 C terminus
Phosphorylation sites on the Thr830 and Ser842 residues of the GluR4 subunit

The GluR3 subunit has a limited number of serine residues on its intracellular C terminal domain (Fig 1.6). So far, phosphorylation of this subunit has not been reported.

GluR3: EFCYKSRAESKRMKLTkNTQNfKPAPATNTQNYATYREGYNVYGTESVKI

Fig 1.7: Aminoacid sequence in the GluR3 C terminus

No phosphorylation sites in the C terminus aminoacidic sequence of the GluR3 subunit

Other mechanisms of phosphorylation of AMPA receptor subunits were suggested. A recent study has demonstrated that the activation of NMDA receptors dephosphorylates serine 845 of the GluR1 subunit in hippocampal slices (Vanhoose and Winder, 2003). This dephosphorylation resulted through the displacement of PKA from synapses by NMDA (Snyder et al., 2005). In addition, activation of phosphatase may contribute to this process because NMDA induced dephosphorylation of serine 845 was blocked by a phosphatase inhibitor (Vanhoose and Winder, 2003)

1.11 AMPA RECEPTOR TRAFFICKING PROTEINS

1.11.1 GRIP-1

Using the yeast two-hybrid system, it has been demonstrated that the carboxyl termini of the GluR2 and GluR3 subunits interact with a 130 kDa protein termed GRIP-1 (Glutamate Receptor Interacting Protein-1) (Dong et al., 1997). GRIP-1 contains seven PDZ domains, but only the fourth and fifth PDZ domains bind to the GluR2 and GluR3 subunits (Fig 1.7). The other PDZ domains do not interact with AMPA receptors and may serve to anchor these receptors to the cytoskeleton or to other regulatory proteins.

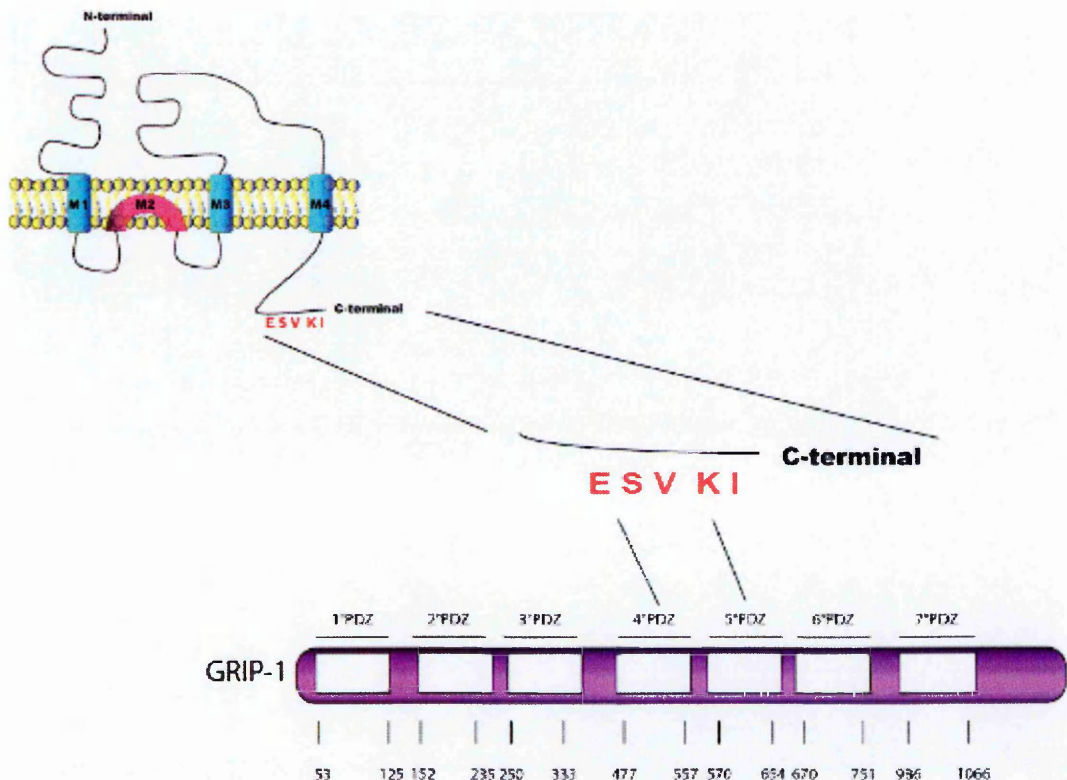


Fig 1.8: GRIP-1 structure and PDZ site interaction in GluR

Interaction of the 4th and 5th PDZ domains of the GRIP-1 with a ESVKI motif in the PDZ region of GluR2 and GluR3 subunits

GRIP-1 interacts with an ESVKI motif in the PDZ region of GluR2 and GluR3 that includes a crucial internal serine and a hydrophobic side chain on the terminating amino acid. GRIP-1 appears to be concentrated at some, but not all, excitatory synapses in cultured neurons.

Studies of subcellular localization by postembedding immunogold electron microscopy have shown in adult rat cortex a considerable synaptic staining and an intracellular labelling within the spines and dendritic shafts. However, a large proportion of GRIP-1 is not associated with excitatory synapse (where AMPA receptors are presumed to be concentrated) but with GABAergic neurons (Wyszynski et al., 1999).

Furthermore, recently it was demonstrated that GRIP-1 is required for the synaptic accumulation of the AMPA receptors. GRIP-1 might accomplish this either by promoting receptor insertion into the synaptic membrane (Osten et al., 2000; Song and Huganir, 2002; Liu and Cull-Candy, 2005) or by anchoring it to the cytoskeleton through the PDZ domains (Braithwaite et al., 2002; Song and Huganir, 2002). In addition, a recent study has reported that GRIP-1 binds to kinesin 5 (KIF5, a microtubule-based motor protein that is important for vesicular transport to axons and dendrites) causing a complex that appears to be involved in the transport of GluR2 dendrites (Setou et al., 2002).

1.11.2 ABP

ABP, is a post-synaptic density protein of 80 KDa related to GRIP-1 with two sets of three PDZ domains. It differs from GRIP-1 notably in lacking the C-terminal seventh PDZ domain. The C-terminal sequence ESVKI shared by AMPA receptor GluR2/3 subunits is reported to bind selectively to the third, fifth and sixth PDZ domains of ABP (Srivastava et al., 1998) (Fig 1.8).

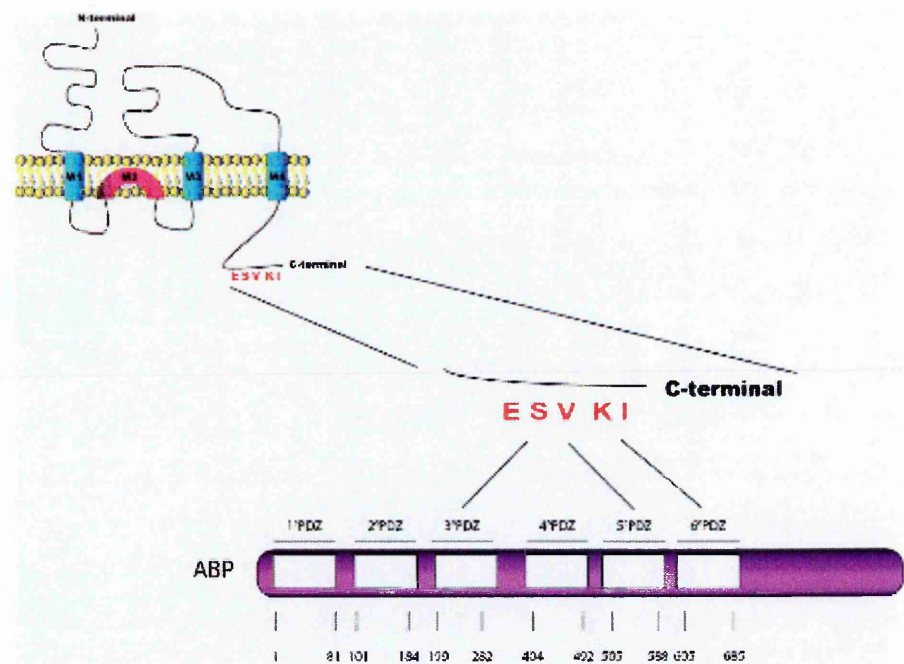


Fig 1.9: ABP structure and PDZ site interaction in GluR

Interaction of the 3rd, 5th and 6th PDZ domains of the ABP with a ESVKI motif in the PDZ region of GluR2 and GluR3 subunits

It exhibits a widespread CNS expression and has been demonstrated to interact in a similar way to GRIP-1 with the GluR2 and 3 AMPA receptor subunits (Srivastava et al., 1998; Srivastava and Ziff, 1999). Furthermore, examining the subcellular distribution of ABP by electron microscopy has confirmed a high localization in the PSD fraction (Srivastava et al., 1998). ABPs can multimerize among themselves or with GRIP. A variant of the ABP, termed ABP-L/GRIP2 protein, has been characterized as a splice variant of ABP containing an N-terminal extension of 52 aa, an internal deletion of 41 aa between PDZ3 and PDZ4, and a C-terminal extension that contains an additional seventh PDZ domain. GRIP-2 has the greatest similarity also with GRIP-1, sharing a highest homology for the sixth PDZ domain (Wyszynski et al., 1999). There is not a definitive explanation about the role of ABP and GRIP in the AMPA receptor trafficking. It has been demonstrated by a mutant GluR2 constructs that could not bind GRIP/ABP that the internalized receptors are stabilized in an intracellular pool by these proteins, preventing them from recycling back to the plasma membrane or to a degradative pathway (Braithwaite et al., 2002). This was confirmed also by electrophysiological results using a peptide that interferes with the GluR2-GRIP/ABP interaction, which was able to cause a run-up of excitatory postsynaptic currents due to the cycle back to the synaptic membrane of the subunits (Daw et al., 2000). In contrast, mutating the GRIP/ABP binding domain caused a decrease in the surface expression of a myc-tagged GluR2 construct expressed in hippocampal cultures by using Sindbis virus mediated gene delivery (Osten et al., 2000). Another study in hippocampal slices cultures using Sindbis virus to express mutant forms of GluR2 observed that synaptic delivery of GluR2 appeared to be completely

prevented by mutations that interfered with binding to NSF or GRIP/ABP (Shi et al., 2001)

1.11.3 PICK-1

PICK1 was originally isolated due to its interaction with protein kinase C (PKC α) (Staudinger et al., 1995; Staudinger et al., 1997) by the yeast two hybrid system as a protein of 416 aa with a molecular weight of 46.5 KDa. In the PICK1 protein sequence was identified a PDZ domain that interacts with a corresponding PDZ-binding domain QSAV at the extreme COOH terminus of PKC α (Staudinger et al., 1997) (Fig 1.9).

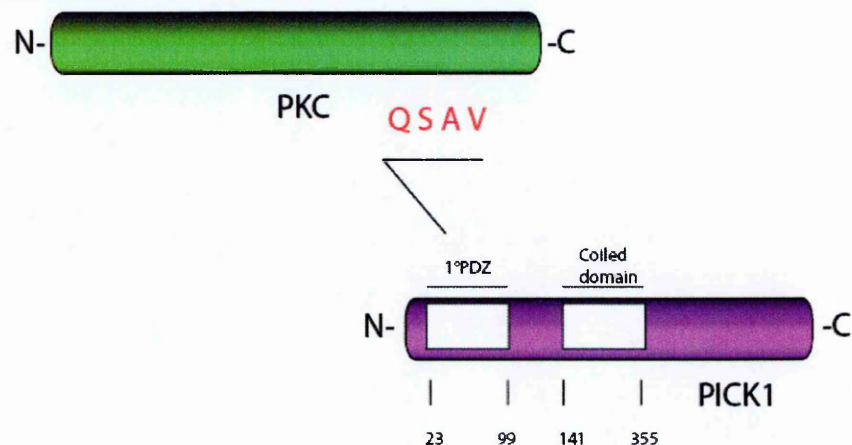


Fig 1.10: PICK1 structure and PKC interaction

Interaction of the PDZ domain of the PICK1 with a QSAV motif in the aminoacidic sequence of PKC

PICK1, unlike GRIP and ABP, has only one PDZ domain that can interact with the AMPA receptor subunits or PKC α , but contains a coiled domain that could potentially be involved in the dimerization of PICK1 interacting with several proteins containing PDZ domains. PICK-1 mRNA was demonstrated to have an ubiquitous distribution, with the highest expression in the brain and testis (Staudinger et al., 1995). A study of distribution at subcellular level in transiently transfected COS cells showed that PICK1 is located in the perinuclear region, the rough endoplasmic reticulum and Golgi apparatus (Staudinger et al., 1995), but it was colocalized also with excitatory synapses (Xia et al., 1999). PICK1 is an efficient substrate for PKC phosphorylation both in vivo and in vitro, but the PKC α binding and phosphorylation of PICK1 appear not to be correlated with the activity since PKC α binds phosphorylated and unphosphorylated PICK1 with the same abilities. It was also speculated that the function of PICK1 is to localize activated PKC α to the plasma membrane, thereby bringing the kinase in proximity with specific substrates (Staudinger et al., 1997). Noteworthy, AMPA receptors are known to be regulated by phosphorylation and PKC is a strong candidate kinase for mediating at least part of the changes in receptor phosphorylation (McGlade-McCulloh et al., 1993; Roche et al., 1994).

A recent study has demonstrated an interaction between the AMPA receptor subunits and PICK1 through PDZ domains. The interaction occurs within the last ten amino acid residues containing a PDZ binding motif (E S V:I K I) of the short C-terminal alternative splice variants of AMPA receptor subunits. However, no interaction was revealed by the authors with the corresponding long splice variants which do not contain the E S V:I K I motif (Dev et al., 1999) (Fig.1.10).

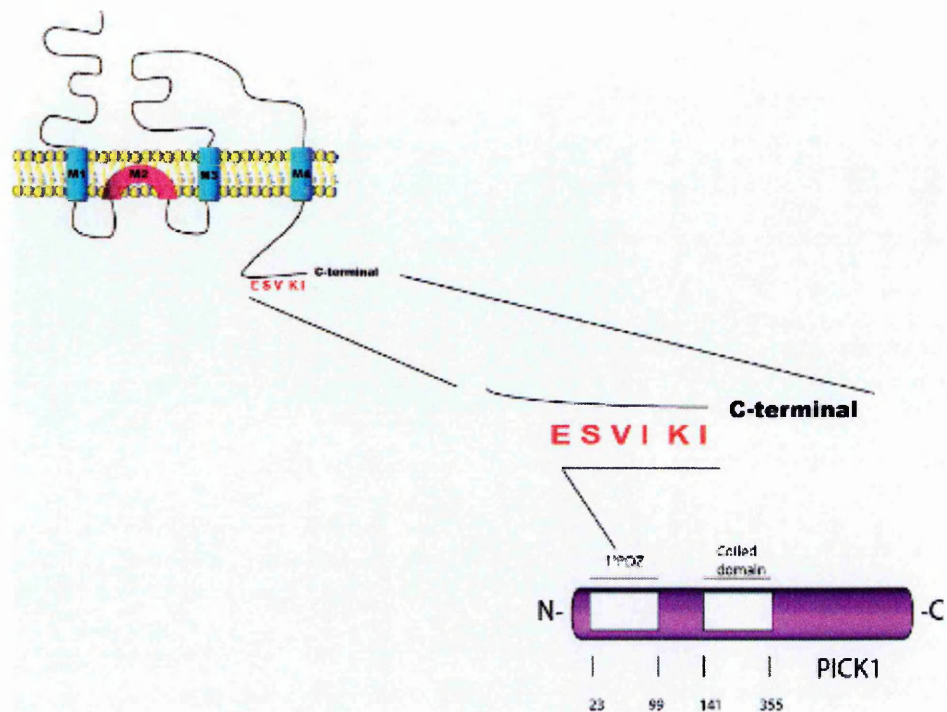


Fig 1.11: PICK1 structure and PDZ site interaction in GluR

Interaction of the PDZ domain of the PICK1 with a ESVIKI motif in the PDZ region of GluR2 and GluR3 subunits

A role for PICK1 as anchoring protein for GluR short subunits to intracellular membrane associated PKC α was proposed, conferring substrate specificity for the phosphorylation event of the AMPA receptors (Dev et al., 1999).

Two mechanisms were hypothesized in the trafficking of the AMPA subunits for PICK1 in Purkinje cultured cells. Assuming that ABP/GRIP is bound in the basal state, the GluR2/3 activation by the PKC responsible for Long Term Potentiation (LTP) induction results in the phosphorylation of GluR2 Ser880. This causes the unbinding of GRIP/ABP and the recruitment of PICK1 to the

Synaptic GluR2/3 containing AMPA, which is then primed for internalization by clathrin-mediated endocytosis (Fig.1.11).

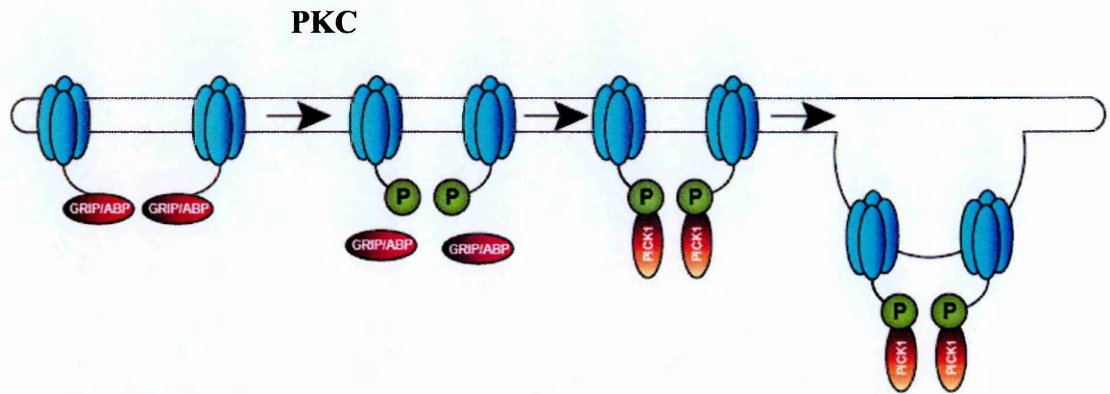


Fig 1.12: 1° mechanism proposed for AMPA trafficking and PICK1

Unbinding of GRIP1/ABP by phosphorylation in C terminal of the GluR subunits, which is mediated by PKC, recruits PICK1 to the GluR subunits priming the clathrin-mediated endocytosis

In another proposed mechanism, GRIP/ABP unbinding is sufficient to prime synaptic AMPA receptors for internalization. PICK1 binds AMPA receptors in the internal pool, stabilizing them and attenuating compensatory AMPA receptor insertion in the membrane (Xia et al., 2000) (Fig.1.12).

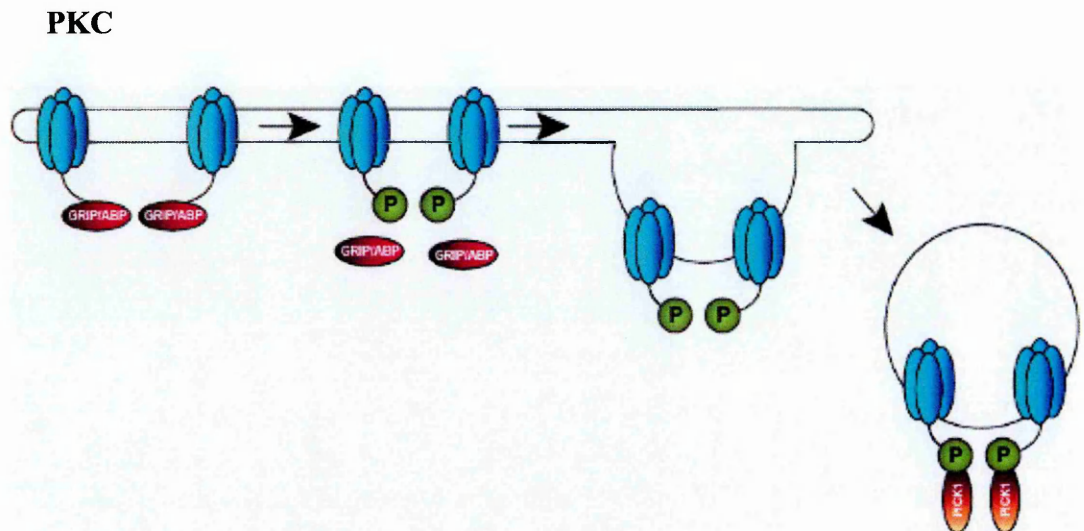


Fig 1.13: 2° mechanism proposed for AMPA trafficking and PICK1

Unbinding of GRIP1/ABP by phosphorylation in C terminal of the GluR subunits, which is mediated by PKC subunits, primes the clathrin-mediated endocytosis. Whereas PICK1 stabilizes the internal pool of them

Furthermore, in NIH3T3 cell culture it was demonstrated that the expression of PICK-1 has a role in the phosphorylation of the AMPA receptors by the protein kinase C (PKC), reducing the surface expression of them (Perez et al., 2001). Another study confirmed a role of PICK-1 in AMPA receptors endocytosis, demonstrating that cells loaded with a peptide that disrupts the GluR2-PICK-1 interaction cause an increase in synaptic response and inhibited LTD (Kim et al., 2001). However, a different role in an opposite movement of the AMPA receptor subunits from intracellular stores to the synaptic membrane was

suggested for PICK-1 and PKC in an electrophysiological study in the hippocampus (Daw et al., 2000).

1.11.4 NSF

NSF was first described in 1998 as a protein involved in membrane fusion events (Block et al., 1988). The step required for bilayer fusion of the vesicle with the target is facilitated by integral membrane proteins called soluble NSF attachment protein receptors (SNAREs). The cytoplasmic domains of these SNAREs form a stable coiled-coil complex that bridges the fusing membranes (trans configuration) and promotes fusion. Once fusion is complete, the SNARE complexes, originally spanning the two membranes, now reside in the same membrane (cis configuration). The cellular role of NSF is to disassemble these cis complexes so SNARE monomers can be recycled and vesicular transport is able to continue. NSF completes this function through an adaptor protein called SNAP (soluble NSF attachment protein), which binds to the SNARE complex and subsequently binds to and stimulates the ATPase activity of NSF (Rothman, 1994).

NSF acts as hexamer (Fleming et al., 1998) and through an ATP hydrolysis provides a driving force that dissociates this complex.

Each promoter is divided into three discrete domains and each domain is connected by a protease sensitive linker region. There is an amino-terminal NSF-N domain (1–205) followed by two ATP-binding domains NSF-D1 (206–477) and NSF-D2 (478–744) (Fig.1.13).

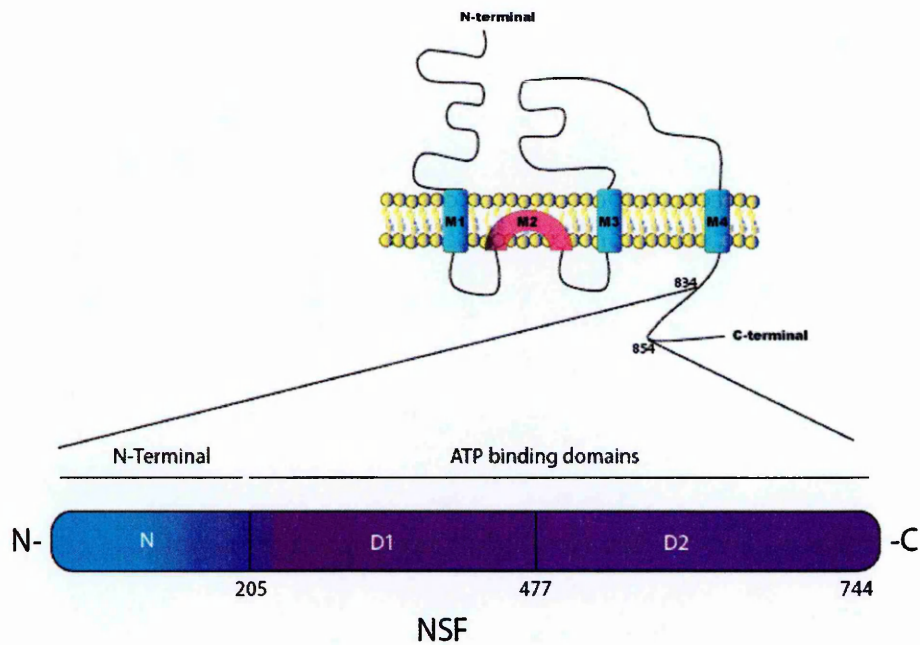


Fig 1.14: NSF structure and no PDZ site interaction in GluR

NSF interacts specifically with no PDZ domain (starting from 834 to 854 residue) in the C-terminus of the GluR2 subunits

It was determined at the ultrastructural level that NSF immunoreactivity is present in large dendritic shafts and in dendritic spines, as well as in axon terminals in the close vicinity of the postsynaptic membrane (Osten et al., 1998).

Different evidences have suggested an important role of NSF also as a stabilizer of AMPA receptors in the plasma membrane, making them resistant to regulated endocytosis (Nishimune et al., 1998; Osten et al., 1998; Noel et al., 1999).

It was demonstrated that NSF interacts exclusively with no PDZ C-terminal site of GluR2 subunit. This region was found to lie within a 21 aa sequence, starting

from 834 to 854 residue (Osten et al., 1998). The presence of this NSF interaction domain seems to be specific for the C terminus of GluR2, as compared with other AMPA receptor subunits (Nishimune et al., 1998; Beretta et al., 2005).

Several models were proposed as functional models of NSF on the GluR2 subunits:

It was demonstrated that NSF and α - and β -SNAPs interact with GluR2 in a complex that in several aspects resembles the interaction of NSF and SNAP at the SNARE complex. The interaction of NSF and SNAP with the AMPA receptor could involve the disruption of multiprotein complexes, such as those formed between the membrane-inserted receptor and the proteins of the postsynaptic density (such as GRIP or ABP) (Hanley et al., 2002). Furthermore, it has been demonstrated that NSF regulates selectively the interaction of GluR2 and PICK1, disassembling PICK1-GluR2 complexes, resulting in an increased incidence of AMPA receptors at the cell surface (Hanley et al., 2002).

The role of the NSF in the AMPA receptors trafficking was studied by using peptides able to interfere with the interaction between GluR2 and NSF. These experiments have demonstrated a fairly rapid decrease in synaptic strength in cell culture (Nishimune et al., 1998; Song et al., 1998; Luscher et al., 1999; Noel et al., 1999), whereas with chronic exposure an almost complete block of trafficking of the AMPA receptors to the surface has been confirmed by electrophysiological observation (Luscher et al., 1999; Noel et al., 1999).

These results suggest that NSF is primarily important when synapses are stimulated, because only the regulated, but not the constitutive cycling of GluR2 was affected by mutating its NSF binding domain.

Other proteins were characterized to regulate the trafficking of the AMPA receptor subunits to the membrane such as **Stargazing**, a PDZ protein able to transfer and maintain the GluRs subunits into the membrane (Yamazaki et al., 2004; Vandenberghe et al., 2005); whereas **4.1 family proteins** are able to interact with GluR1 and 4, controlling the transport and the stabilization to the membrane (Shen et al., 2000; Coleman et al., 2003).

NARP (neuronal activity regulated pentraxin), a presynaptic protein, instead plays an important role in the receptors aggregation to the cells surface. It has been also showed that the editing state FLIP/FLOP and at the Q/R site of the GluRs subunits influences the trafficking to the membrane surface. The expression of receptors containing both the FLIP and FLOP isoform is able to reach more easily the membrane than the homomeric receptors (Brorson et al., 2004). Furthermore, the unedited form at the Q/R site is much more efficiently transferred to the membrane than the edited form (Greger et al., 2002; Greger et al., 2003)

1.12 PATHOGENIC HYPOTHESES OF ALS

Different hypotheses were proposed to explain the cause of ALS pathology. The critical point, as in other neurodegenerative diseases, is the explanation of the selective vulnerability of a given cell population, namely the motor neurons in ALS.

1.12.1 OXIDATIVE DAMAGE

The role of oxidative stress as a primary or secondary event in the pathogenesis of ALS still remains controversial. Increase of oxidative damage markers such as protein carbonyl adducts, lipid peroxidation and DNA damage, were reported in human patients affected by both sporadic and inherited forms of ALS (Beal et al., 1997; Ferrante et al., 1997b; Liu et al., 1999) and in a transgenic mouse model of the disease (Ferrante et al., 1997a; Andrus et al., 1998; Liu et al., 1998). However, in other studies, no significant differences in markers of oxidative damage associated with the expression of SOD1 mutants were found (Bruijn et al., 1997a). Evidence of increased oxidative insult was provided more consistently in sporadic ALS patients (Shaw et al., 1995a; Ferrante et al., 1997b; Pedersen et al., 1998).

Concerning the familial forms of ALS and SOD1 mutant transgenic mice, it was hypothesized that a possible source of oxidative insult may be represented by the gain of toxic function of mutated SOD1 (Cleveland and Rothstein, 2001). Yim and co-workers showed that mutations of SOD1 protein can decrease its

K_m for hydrogen peroxide (Yim et al., 1996; Yim et al., 1997). The use of hydrogen peroxide as a substrate by the reduced SOD1-Cu⁺ form might produce the extraordinarily reactive hydroxyl radical (OH[•]) leading to a cascade of peroxidation (Wiedau-Pazos et al., 1996). Singh and colleagues demonstrated that a significant fraction of 5,5'-dimethyl-1-pyrroline N-oxide (DMPO)/OH formed during the reaction of SOD1 and familial ALS SOD1 mutants with hydrogen peroxide (H₂O₂) is derived from the incorporation of oxygen from water due to oxidation of DMPO to DMPO/OH, presumably via DMPO radical cation. However, no differences were detected between wild type and mutant form of SOD1 (Singh et al., 1998).

Beckman and collaborators proposed that an altered folding of the enzyme caused by the mutations, might determine a greater access of abnormal substrates such as peroxynitrite (ONOO⁻) to the catalytic copper site. This could lead to aberrant tyrosine nitration and numerous toxic events (Beckman et al., 1993).

Another hypothesis suggests that mutations may reduce the amount of bound zinc, allowing a rapid reduction of mutant SOD1 to the Cu¹⁺ form by abundant intracellular reductants. The reduced SOD1 mutant would then run the normal catalytic step backwards, converting oxygen to superoxide. The superoxide so produced would react with nitric oxide producing peroxynitrite, which would promote intracellular damage, including protein nitration (Estevez et al., 1999).

The time course of accumulation of oxidative damage relative to disease onset and progression in ALS patients is not known. Even if oxidative damage is secondary and does not initiate toxic events, it is probable that oxidative stress contributes significantly to the neuronal death in ALS. Treatment with the antioxidant vitamin E (alpha-tocopherol) slowed down the onset and progression of paralysis in transgenic mice expressing SOD1 carrying the G93A

mutation (Gurney et al., 1996). However, a clinical trial carried out on ALS patients with the same drug did not ameliorate survival or motor function (Desnuelle et al., 2001).

1.12.2 MITOCHONDRIAL ALTERATIONS

Several lines of evidence suggest that mitochondrial dysfunction may be involved in the pathogenesis of ALS. Studies in human postmortem and biopsy samples have described mitochondrial abnormalities in proximal axons (Hirano et al., 1984b) and in the anterior horns of sporadic ALS spinal cords (Sasaki and Iwata, 1996). Subsarcolemmal aggregates of abnormal mitochondria were found in intramuscular nerves and skeletal muscle (Afifi et al., 1966; Atsumi and Miyatake, 1987), which also showed increased mitochondrial volume and calcium levels (Siklos et al., 1996). Furthermore, defects in the activities of mitochondrial respiratory chain complexes were detected in muscle and spinal cord of sporadic ALS patients (Wiedemann et al., 1998; Borthwick et al., 1999; Vielhaber et al., 2000; Wiedemann et al., 2002) suggesting that respiratory chain impairment may participate in the pathogenesis of SALS. Moreover, a decrease of COX activity was recently reported in motor neurons and muscle biopsies of sporadic ALS patients, suggesting a role in the mechanism of the disease (Borthwick et al., 1999).

Massive vacuolization of cytoplasm and swollen mitochondria were observed in the motor neurons of transgenic mice lines carrying the G93A or G37R mutation at the early stage of the disease (Dal Canto and Gurney, 1995; Wong

et al., 1995; Bendotti et al., 2001a), suggesting that mitochondrial pathology is an early preclinical feature of motor neurons damage in these mice.

There is others evidences indicating that in the same mice a reduced amount of respiratory enzymes (complexes I and IV) in spinal cord and brainstem was present (Browne et al., 1998). More recently different studies have reported an accumulation of mutant SOD1 in the mitochondria of the spinal cord from SOD1G93A mice (Higgins et al., 2002; Liu et al., 2004). These findings support the hypothesis that mutant SOD1 may damage mitochondrial function and integrity directly from inside the mitochondria. Furthermore, mitochondrial alterations seem to be the early event in the vacuolization of the motor neuron in transgenic mice. The vacuoles are belived to be formed by the expansion of mitochondrial outer membrane which can be fused with membranes of other subcellular organelles (Bendotti et al., 2001a; Higgins et al., 2003). Moreover, SOD1 mutations not only cause mitochondrial morphological alterations, but also result in mitochondrial dysfunction, both in animal and cellular models of FALS. A significant loss of mitochondrial membrane potential, which is an indicator of mitochondrial respiratory chain dysfunction have been documented in neuroblastoma cells expressing mutant SOD1 (Carri et al., 1997). In addition, a decrease of ATP levels, impaired respiratory chain enzymes, and increased production of reactive oxygen species were demonstrated in motor neuron-like cells and neuronal cell lines expressing mutant SOD1 (Menzies et al., 2002; Beretta et al., 2003).

In SOD1G93A mice at an early disease stage, the activities of various mitochondrial electron transfer chain complexes were decreased in the spinal cord. This decrease persisted throughout the course of disease progression (Jung et al., 2002).

Additionally, indirect evidences that mitochondrial energy metabolism is impaired by mutant SOD1 was supported by the fact that administration of creatine, a molecule involved in energy storage, increases the lifespan of G93A mice (Klivenyi et al., 1999). A decrease in several respiratory chain complexes and in the capacity to consume oxygen and synthesize ATP have been also revealed in isolated mitochondria from brain and spinal cord of G93A mice (Mattiuzzi et al., 2002).

Another group using isolated mitochondria from presymptomatic and symptomatic G93A mice has demonstrated that G93A mitochondria suffer from a loss of cytochrome *c* associated with the inner mitochondrial membrane leading to altered mitochondrial respiration (Kirkinezos et al., 2005). Furthermore, it was demonstrated that the elevation of the cytosolic calcium levels in neurons induces enhanced production of free radicals from mitochondria, and compromises mitochondrial integrity (Reynolds, 1999), although the mechanism underlying this damage is still unclear. Consistent with this evidence, Carri et al. showed that neuroblastoma cells transfected with mutant SOD1 displayed increased cytoplasmic calcium levels, indicating a reduced calcium-buffering capacity (Carri et al., 2003).

A trigger stimulus to cell death by necrosis or apoptosis is the permeabilization of the mitochondrial membranes that can be induced through activation of the mitochondrial permeability transition pore (MPTP) (Kroemer and Reed, 2000). The MPTP is a voltage-gated cation-permeable channel, whose opening is favored by several factors including depolarization, intramatrix Ca^{2+} accumulation and oxidizing agents like reactive oxygen species. All these can be altered in the motor neurons by the presence of mutated SOD1.

Interestingly, it was demonstrated that the altered mitochondria appeared first in dendrites of the anterior horn of the spinal cord, than in cell bodies. Thus, it is possible that a synergistic effect between glutamatergic stimuli and the toxic effect of the SOD1 mutant in motor neurons may induce activation of the MPTP. This in turn would be able to permeabilize the mitochondrial membranes, with consequent mitochondrial osmotic changes and membrane swelling, and this may be the initial step of the cascade of events leading to motor neuron death. Consistent with this hypothesis is the finding that overexpression of Bcl2, an antiapoptotic factor, which is able to inhibit the opening of MPTP, delays the disease onset and prolongs survival in transgenic mice carrying mutated SOD1 (Kostic et al., 1997). Furthermore, treatment with coenzyme Q and creatine, both inhibitors of the MPTP, significantly prolonged the life span of transgenic mice carrying the G93ASOD1 mutant (Matthews et al., 1998; Klivenyi et al., 1999).

1.12.1.3 CYTOSKELETAL ABNORMALITIES

An important hallmark in the ALS pathology is the intermediate filament accumulation (IF). There are three major IF types expressed in adult neurons: neurofilaments, α -internexin and peripherin. Neurofilaments (NF) are the most abundant neuronal IF proteins, being expressed in most neurons of the central (CNS) and peripheral (PNS) nervous systems (Julien and Mushynski, 1998; Gotow, 2000). They comprise three protein subunits, NF-L of 68–70 kD, NF-M of 150–160 kD and NF-H of 200–210 kD. Alpha-internexin, a 66-kD protein, is expressed mainly in the CNS (Pachter and Liem, 1985). Peripherin, a 58-kD

protein, is expressed mostly in the PNS and in some neuronal populations of the CNS (Portier et al., 1983; Parysek and Goldman, 1988).

These alterations were revealed in both sporadic and familial ALS (Hirano et al., 1984a; Hirano et al., 1984b) and in transgenic SOD1 mice (Julien et al., 1998; Robertson et al., 2002). Small in frame deletions or insertions in the tail domain of the NFH neurofilament subunit were reported to occur in 1% of sporadic ALS (Al-Chalabi et al., 1999). However, these alterations do not seem to provoke by themselves motor neuron disease. In order to evaluate if neurofilament alterations is a key factor in the ALS pathology, transgenic mice with mutant neurofilament subunits have been developed. Several models have been generated as an increase gene expression of NF-L (Neurofilament Light) or NF-H (Neurofilament High). Cross breeding with SOD1 transgenic mice slow down the progression of the disease through an accumulation of neurofilament in the cytoplasm rather than in the proximal axon, the latter being considered an important event in the block of the axonal transport. This is likely due the neurofilament acting as a sink against harmful stimulus in the cell body (Couillard-Despres et al., 1998). However, other types of intermediate filament aggregates seem neurotoxic, such as those found in transgenic mice overexpressing either peripherin or an assembly-disrupting NF-L mutant (Beaulieu and Julien, 2003). However they do not appear to be particularly important in ALS motor neuron degeneration (Lariviere et al., 2003).

1.12.4 PROTEIN AGGREGATION

One consistent neuropathological feature of both the sporadic and familial forms of ALS is the presence of protein aggregation (Leigh et al., 1991; Bruijn et al., 1998; Bendotti et al., 2004). Aggregates have also been revealed in the

motor neurons and in the astrocytes of SOD1G85R mice before the onset of clinical symptoms (Bruijn et al., 1997b), in SOD1G93A mice as a first neuropathological sign (Johnston et al., 2000; Cheroni et al., 2005) and in cell culture model system (Bruijn et al., 2004). One of the most represented proteins in the aggregate is SOD1 in an insoluble high molecular weight form (Johnston et al., 2000; Cheroni et al., 2005). The SOD1 aggregates have been classified in two classes. The first is represented by aggregates detectable by conventional histological stains and are characterized by intense and localized SOD1 immunoreactivity throughout the inclusions or, less frequently, only at the periphery of them. The second type includes cell body deposits that are more diffusely distributed. Misfolded SOD1 aggregates can not be dissociated, even with strong detergents and reducing agents. Their harmful effects could result from altered catalytic activity mediated by the misfolded aggregated mutants, the cosequestering of essential cellular proteins such as chaperones and/or overload of the ubiquitin proteasome pathway, which degrades damaged proteins (Cleveland and Rothstein, 2001). Another aggregated protein revealed in human and ALS transgenic mice is the ubiquitin (Stieber et al., 2000; Watanabe et al., 2001; Bendotti et al., 2004). This evidence has lead some workers to think that the proteasome machinery is implicated in this pathology, given the role of ubiquitin in the degradation process through the proteasome (Kabashi et al., 2004; Cheroni et al., 2005). Another protein revealed accumulated in human and transgenic mice tissue is p38MAPK (Tortarolo et al., 2003; Bendotti et al., 2004). This protein is linked to intracellular mechanisms of death and it was demonstrated to be able to phosphorylate neurofilament and activate transcription factors which in turn lead to cytokine expression. Furthermore, phosphorylated neurofilaments and dorfins, a RING-IBR type

ubiquitin ligase (E3) which can ubiquitinate mutant SOD1, were characterized in abnormal intracellular perikaryal aggregates (Bendotti et al., 2004; Ishigaki et al., 2004).

However, it is not yet clear what the role is of these aggregates in the disease pathogenesis, or whether they are harmless by-products, or potentially beneficial through the sequestration of abnormal proteins.

1.12.5 INFLAMMATION

A clear evidence of an involvement of inflammation events characterizes the pathology of ALS. Activation and proliferation of microglia was found in regions of motor neuron loss, such as the primary motor cortex, brainstem motor nuclei, corticospinal tracts and the ventral horns of the spinal cord (Ince et al., 1996). Furthermore, post-mortem studies of familial and sporadic ALS revealed that activated microglia occurred not only in areas of severe motor neuron loss, but also in regions where there was only mild motor neuron damage (Ince et al., 1996). This suggests that microglia are activated early in the pathogenesis of human ALS. This was supported by the evidence of an increased binding of peripheral benzodiazepine, a marker for activated microglia, in the brain of ALS patients at the onset of the disease as detected by positron emission tomography (PET scan) (Turner et al., 2004). It was also documented that an activation of microglia and astrocytes characterize the progression of the disease of the transgenic mice already at the presymptomatic stage of the disease (Kawamata et al., 1992; Hall et al., 1998; Sargsyan et al., 2005). However, an elegant work recently published has confirmed an involvement of the microglia exclusively during the progression of the disease, showing that diminishing selectively the mutant human SOD1 in the microglia of the transgenic mice had little effect on the early disease phase but sharply slowed later disease progression (Boillee et al., 2006).

Furthermore, several studies have also demonstrated an increase expression of proinflammatory mediators (TNF- α , IL6) in the serum of ALS patients (Sekizawa et al., 1998; Poloni et al., 2000) and increased levels of TNF- α , IL-1b, IL-6 TGFbeta, iNOS and COX-2 in ALS transgenic mice and rat, even

preceding the development of clinical signs (Almer et al., 2001; Elliott, 2001; Almer et al., 2002; Weydt et al., 2002; Hensley et al., 2003; Xie et al., 2004). An increase expression of TNFR-1 and 2 was also revealed in the motor neurons of G93A transgenic mice already at the presymptomatic stage of the disease, and becomes particular evident also in the microglial cells at the endstage of the disease (Veglianese et al., 2006).

Recently, a series of studies revealed that the SOD1 mutations are probably not directly deleterious for motor neurons, but exert their neurotoxic effects in a 'non-cell autonomous' mode involving non-neuronal cells in the degeneration of the motor neurons. Indeed, it was shown that neither neuronal nor astrocytic expression of the mutated SOD1 transgene is sufficient to produce motor neuron degeneration in mice (Gong et al., 2000; Pramatarova et al., 2001; Lino et al., 2002). Furthermore, Clement et al. showed that in mutated SOD1 chimeric mice (mice with mosaic expression of the mutated SOD1 transgene) the severity of the ALS-phenotype depended on the proportion of CNS cells expressing the mutant transgene, but not on the cell type (i.e. neurons vs. nonneurons) (Clement et al., 2003). Individual motor neurons expressing mutated SOD1, but surrounded by non transgenic glia remained healthy, whereas non transgenic neurons bordered by mutant SOD1 expressing glia degenerated. This showed that mutated SOD1 expression in motor neurons is neither sufficient nor necessary to produce motor neuron degeneration.

1.12.6 EXCITOTOXICITY

The condition referred as “excitotoxicity” was formulated for the first time by Olney (Olney, 1990). Experimental evidences have demonstrated that excitotoxicity could contribute to neuronal damage in a wide range of neurological disorders such as seizures, cerebral ischemia, head traumas, Alzheimer’s disease, Huntington’s chorea and AIDS encephalopathy, including ALS. Furthermore, it was demonstrated that specific forms of motor neuron disease are caused by oral intake of excitotoxins. For instance, consumption of mussels containing Domoic acid led to neurological symptoms such as headache, seizures, hemiparesis and motor neuronopathy (Teitelbaum et al., 1990). The excitotoxin BOAA (β -N-oxalyl-amino-L-alanine), a potent AMPA receptor agonist present in the pea *Lathyrus sativus* caused lathyrism, an upper motor neuron disease characterized by spasticity (Spencer et al., 1986; Bridges et al., 1989). In addition, intrathecal injections with BOAA induced motor neuron degeneration in rat and mouse spinal cord (Chase et al., 1985). Another excitotoxin, BMAA (β -methylamino-L-alanine), present in the seeds of *Cycas circinalis* lead to Western Pacific ALS-Parkinsonism dementia (Guam Island). It was demonstrated that BMAA is an NMDA and AMPA receptor agonist (Copani et al., 1991).

Glutamate is the major excitatory amino acid of the central nervous system (CNS) which functions as a chemical signal in nervous conduction. It is located predominantly in the presynaptic vesicles that release glutamate in the cleft through a calcium dependent mechanism. When released, glutamate binds to receptors which are either ionotropic or metabotropic.

Ionotropic receptors are the AMPA, NMDA and Kainate receptors forming ionic channels, while the metabotropic receptors are linked to G proteins that transduce the signal into the cytoplasm. One of the main mechanisms of glutamate inactivation is its re-uptake into astrocytes, where it is metabolized to α -ketoglutarate or converted to glutamine by glutamine synthetase.

Two types of excitotoxicity are distinguished: a **classical excitotoxicity** that occurs after an acute increase of the extracellular glutamate concentration (extracellular concentration of 2-5 μ M is sufficient to cause degenerations of neurons) and is thought to induce damage in conditions such as stroke, status epilepticus and neurotrauma; while **slow excitotoxicity** characterized by a chronic and milder elevation of glutamate are believed to underlie excitotoxicity in neurodegenerative diseases (Van Den Bosch et al., 2006). An increased concentration of glutamate in the extracellular cleft can occur through an augmented release from presynaptic terminals or when the reuptake from the synaptic cleft through the glutamate transporter (EAAT2/GLT-1) located in the astrocytes is insufficient. Elevated concentrations of glutamate can also be released from injured neurons. Increased levels of glutamate in the synaptic cleft determine an excessive influx of calcium through the over-stimulation of its own receptors, both NMDA and AMPA. Furthermore, an augmented activation of kainate receptors is able to lead to the release of calcium from the endoplasmic reticulum (Pellegrini-Giampietro et al., 1997). Different mechanisms can be adopted by the cell to control the intracellular levels of calcium, such as pumping it outside, driving it into intracellular compartments that function as calcium sinks (i.e. mitochondria and endoplasmic reticulum), or providing proteins able to bind it (i.e., parvalbumin and calbindin) (Heath and Shaw, 2002; Arundine and Tymianski, 2003). A deregulated homeostasis of

these defense systems may cause excessive intracellular calcium ion concentration that leads to the activation of cell death pathways. For example, increased levels of calcium in the cytoplasm activates protein kinases, phospholipases, and nitric oxide synthase and produces free radicals which destroy plasma membrane lipids, DNA and proteins (Orrenius and Nicotera, 1994). Furthermore, an excessive cytosolic calcium level may induce activation of the MPTP, with consequent osmotic changes and membrane swelling. This may be the initial step of the cascade of events leading to a failure of the ATP synthesis, reducing the energy available to maintain the physiologic concentration of calcium in the cytoplasm and creating a vicious circle.

For many years the main protagonist associated with events of excitotoxicity was the NMDA receptor, which was considered the only channel able to increase the calcium concentration in cells. Recently another receptor has been demonstrated to be important in the regulation of the calcium entry, particularly during chronic exposure to glutamate, namely the AMPA receptor. In a physiologic state a positively arginine residue in the GluR2 subunit makes the AMPA receptors impermeable to calcium entry, whereas an unedited form of this subunits, with a glutamine in the place of the arginine, makes the receptor permeable to calcium, and therefore able to trigger excitotoxic events.

Several experiments both in vivo and in vitro have demonstrated a selective vulnerability of motor neurons mediated by AMPA receptors. Intrathecal or intraspinal administration of AMPA receptor agonists induced motor neuron degeneration in animals, but this was not seen with an NMDA agonist (Pisharodi and Nauta, 1985; Hugon et al., 1989; Urca and Urca, 1990; Nakamura et al., 1994; Ikonomidou et al., 1996; Kruman et al., 1999). Organotypic rat spinal cord motor neurons cultures were vulnerable to AMPA

receptor mediated excitotoxicity, which could be prevented by antagonists (Rothstein et al., 1993; Saroff et al., 2000). A similar result has been obtained with motor neurons in culture when stimulated with AMPA agonist (Estevez et al., 1995; Urushitani et al., 1998; Vandenberghe et al., 1998; Fryer et al., 1999; Van Den Bosch and Robberecht, 2000). However, other neurons were resistant to AMPA stimulation (Van Den Bosch et al., 2000). To explain this selectivity different hypotheses were formulated, such as low levels of Ca^{2+} buffering proteins (Celio, 1990; Ince et al., 1993; Alexianu et al., 1994). This condition leads the mitochondria to have a more important role in Ca^{2+} buffering in motor neurons. Furthermore, motor neurons have a high proportion of Ca^{2+} permeable AMPA receptors (Carriedo et al., 1996; Van Den Bosch et al., 2000), and it was demonstrated that the Ca^{2+} entry via Ca^{2+} permeable AMPA receptors is responsible for selective motor neuron death and this was confirmed also by AMPA antagonist (Van Den Bosch et al., 2000). Electrophysiological experiments have revealed a higher sensitivity to external polyamines, a lower rectification index and a higher relative Ca^{2+} permeability ratio than other neurons, suggesting a deficiency in GluR2 subunits (Van Damme et al., 2002).

The excitotoxic hypothesis proposed for ALS arises from several studies indicating a disregulated glutamate metabolism in ALS cases. The level of glutamate in the cerebrospinal fluid (CSF) (Rothstein et al., 1990; Shaw et al., 1995b) and in the plasma of ALS patients (Plaitakis and Caroscio, 1987) was found to be significantly elevated, although other studies have not confirmed these evidences (Perry et al., 1990; Camu et al., 1993). Other groups have demonstrated instead a reduced level of glutamate in several brain regions and in the spinal cord of ALS patients (Perry et al., 1987). Furthermore, toxicity was detected when ALS CSF and plasma were used in culture of neurons (Roisen et

al., 1982; Couratier et al., 1993; Cid et al., 2003; Sen et al., 2005), although these data were not confirmed using motor neuron cultures (Iwasaki et al., 1995).

1.12.6.1 GLUTAMATE CLEARANCE HYPOTHESIS

The clearance of glutamate from the extracellular environment by transporter-mediated uptake is an important mechanism to manage the glutamate concentration in the synaptic space. Glutamate transporters are expressed by many cell types in central nervous system, including oligodendrocytes, microglia, astrocytes and neurons (Rothstein et al., 1994). Five types of high-affinity Na⁺-dependent glutamate transporters, designated EAAT1 (known as GLAST in rodents), EAAT2 (named GLT1 in rodents), EAAT3 (EAAC1 in rodents), EAAT4 and EAAT5, have been cloned since 1992 (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992; Arriza et al., 1997). GLT-1 and GLAST are predominantly localized on astrocytes. GLT-1 is the most abundant glutamate transporter in the forebrain, with GLAST particularly expressed in the cerebellum. The majority of glutamate uptake is into astrocytes and is mediated by GLT-1 and GLAST, which are therefore considered the most important in removing glutamate from the synaptic cleft during normal neurotransmission (Rothstein et al., 1996; Danbolt, 2001). An alteration of the glutamate uptake capability in astrocytes was suggested to be the possible cause of excitotoxic motor neuron death in ALS. Any decrease of glutamate uptake by astrocytes can lead to an increase of synaptic glutamate concentration, to overstimulation of glutamate receptors and consequently to excitotoxic insults to motor neurons. Consistent with this hypothesis is a diminished glutamate transport by the astroglial glutamate transporter EAAT2/GLT1 detected in the brain and spinal cord of ALS patients (Rothstein et al., 1992; Shaw et al., 1994; Rothstein et al., 1995). Experiments on organotypic spinal cord with antisense oligonucleotides to EAAT2/GLT1 were demonstrated to induce motor neuron

death (Rothstein et al., 1996). A similar result was obtained when the oligonucleotides was injected by intraventricular administration (Rothstein et al., 1996). A decrease level of this transporter was also described in the ventral horn of SOD1G93A and SOD1G37R mice (Bruijn et al., 1997b; Bendotti et al., 2001b) and in rat mutated in SOD1G93A (Howland et al., 2002). Whereas other studies showed no change in EAAT2/GLT1 transporter (Sasaki et al., 2001; Deitch et al., 2002). Noteworthy, is the oxidative damage of EAAT2/GLT1 through the catalysis of H_2O_2 by mutant SOD1 revealed in vitro (Trotti et al., 2001), suggesting a vulnerability of the glutamate transporter to toxic effect of SOD1. Furthermore, crossbreeding EAAT2/GLT1 overexpressing mice with SOD1G93A mice delayed disease onset, but did not prolong survival (Guo et al., 2003). Recently a blinded screen of 1040 drugs has revealed a β -lactam antibiotic (Ceftriaxone) as a potent stimulator of EAAT2/GLT1 expression, and this was also confirmed in vivo (Rothstein et al., 2005). Mutant SOD1 mice treated with this antibiotic showed an increased lifespan of about 11% and prevented motor neuron degeneration and astrogliosis (Rothstein et al., 2005). Taken together these data indicate that a higher clearance of glutamate from the synaptic cleft can ameliorate the ALS pathology.

1.12.6.2 ROLE OF AMPA RECEPTORS

As described previously, it is quite well established that motor neurons are more susceptible than other spinal neurons to toxicity mediated by AMPA receptors. It was hypothesized that this selective vulnerability of motor neuron to AMPA activation, in respect to other spinal neurons, might result from differences in

AMPA receptor subunit expression. For example, the presence of AMPA receptors lacking GluR2 or lacking edited GluR2 would confer an increased Ca^{2+} permeability, which in turn can lead to deleterious enhanced levels of intracellular calcium and cell death. A decrease of GluR2 and GluR1 mRNA were found in spinal cord homogenate of ALS patients in comparison to controls (Virgo et al., 1996; Takuma et al., 1999). However, a more accurate experiment by real time PCR did not reveal differences in the levels of GluR2 mRNA between ALS patients and control groups (Kawahara et al., 2003), whereas a lower GluR2 editing efficiency was found in spinal grey matter of ALS sporadic patients (Takuma et al., 1999). This phenomenon was subsequently confirmed in single motor neurons isolated by laser microdissection from ALS sporadic patients (Kawahara et al., 2004b).

This Q/R site editing in GluR2 occurs with virtually 100% efficiency in mammalian neurons from an embryonic stage throughout life. This editing is catalyzed transcriptionally by the enzyme adenosine deaminase acting on RNA type 2 (ADAR2) where a glutamine (Q) codon of GluR2 is substituted by an arginine (R) codon through the editing of adenosine (A) to inosine (I) in the RNA.

Therefore, AMPA receptors that contain at least one edited GluR2 subunit have low Ca^{2+} conductance, whereas those lacking a GluR2 subunit or containing an unedited GluR2 subunit are Ca^{2+} permeable.

A significant downregulation of the flop form of GluR2 in the flip/flop region (a small aminoacidic segment preceding the fourth transmembrane region that impart different pharmacological and kinetic properties encoded by mRNA splice variants) was also observed, suggesting the formation of slowly

desensitizing AMPA receptors could be an important mechanism of increase calcium entrance (Tomiyama et al., 2002).

In contrast to ALS patients, no changes in GluR2 editing was found in SOD1G93A rats (Kawahara et al., 2006).

However, recent results from our group have shown a reduction in the GluR2 subunit protein level in motor neurons of SOD1G93A mice already at the presymptomatic stage of the disease, suggesting a likely causal role in the mechanism of motor neuron death. Since the GluR2 mRNA levels were unchanged, it has been hypothesized that a post translational modification was involved in reducing the levels of this subunit (i.e. increased protein degradation) (Tortarolo et al., 2006).

In line with the hypothesis of a role of AMPA receptor in motor neuron degeneration, in vivo treatment of SOD1G93A mice with AMPA receptor antagonists RPR119990 (Canton et al., 2001), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzoxoquinoline (NBQX) (Van Damme et al., 2003) and ZK187638 (Tortarolo et al., 2006) prolong the lifespan of these mice respectively by 13%, 10% and 11%. Moreover, blocking the carboxypeptidase II with 2-MPPA (necessary for the metabolism of glutamate) increased the survival of SOD1G93A mice (Ghadge et al., 2003).

All these data suggest that AMPA receptors can be crucially involved in the onset and the progression of the disease in SOD1 mutant mice. It is still unclear, however, how they can be regulated to determine the selective vulnerability of spinal motor neurons.

A tight regulation of the AMPA receptor trafficking by several proteins (Braithwaite et al., 2000; Ehlers, 2000; Luscher et al., 2000; Man et al., 2000; Turrigiano, 2000; Carroll et al., 2001) and phosphorylation of the AMPA

receptor subunits by several kinases (Matsuda et al., 1999; Chung et al., 2000; Boehm and Malinow, 2005) were recently characterized as a determining regulatory mechanism of AMPA receptor function. A possible deficiency of these mechanisms regulating the bidirectional trafficking of the AMPA subunits between the cytoplasm and the postsynaptic membrane, may represent important targets that if compromised may lead to motor neuron degeneration.

1.13 AIM OF THE STUDY

The aim of this thesis is to carry out a study that may allow us to understand further the mechanisms that trigger or contribute to the development of ALS pathology. In particular, I focussed attention on the characterization of the role of the AMPA receptor in the selective spinal motor neuron death in FALS mice.

Specific aims of this thesis are:

- To further define the selective changes of AMPA receptor subunits in the vulnerable spinal motor neurons of SOD1G93A mice retrogradely labelled with fluorogold injected into the hind limb muscles.
- To investigate whether an altered composition of the AMPA receptor subunits in the post-synaptic membrane, where they are functional active, and in the cytosol, where they are stored, might determine an early predisposition to the excitotoxicity.
- To study the phosphorylation state of the GluR2 subunit, the most important subunit able to regulate calcium entry into neuronal cells, in SOD1G93A mice as a further mechanism of regulation of the trafficking of this subunit between the membrane and cytosol.

- To evaluate whether AMPA receptor trafficking proteins (ABP, PICK1 and NSF), which are able to regulate AMPA receptor subunit trafficking and degradation, are altered in their distribution and expression in SOD1G93A mice. This could explain a possible deregulated recycling of the AMPA receptor subunits previously analyzed.

CHAPTER 2

METHODS

2.1 ANIMALS

Procedures involving animals and their care were conducted in accordance with the institutional guidelines that are in compliance with national (D.L. no. 116, G.U. suppl. 40, Feb. 18, 1992, Circolare No.8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 DEC.12, 1987; NIH Guide for the Care and use of Laboratory Animals, U.S. National Research Council, 1996). The animals were housed under standard conditions ($22 \pm 1^\circ\text{C}$, 60% relative humidity, 12 hour light/dark schedule), 3-4 per cage, with free access to food (Altromin, MT, Rieper) and water.

Transgenic mice, expressing about 20 copies of mutant human SOD1 with a Gly 93 Ala substitution (SOD1G93A), were originally obtained from Jackson Laboratories (B6SJL-TgNSOD-1-G93A-1Gur). Male SOD1G93A mice were repeatedly backcrossed with non transgenic female C57BL/6 mice obtaining transgenic mice on the homogeneous C57BL/6 genetic background. These mice develop the first signs of neuropathology at the motor neuronal level around one month of age while the first symptoms of muscular dysfunction appear around three months of age, with tremors and a progressive reduction in the extension reflex of the hind limbs, when the mice are raised by the tail. At about four months of age, the mice start to show a progressive muscular weakness, revealed by the increasing difficulty to stay on a rotating bar and by a reduction in stride length on an inclined ramp. At this stage, more than 50% of motor neurons of the lumbar spinal cord are lost and two months later these mice die (Ciavarró et al, 2003).

Mitochondrial vacuolisation and the swelling of motor neurons are among the earliest events and are accompanied by a decreased function of the mitochondria

(Bendotti et al., 2001). Later, but still at the asymptomatic stage, the SOD1G93A mice show signs of cytoskeletal disorganization in the motor neurons, with the accumulation of phosphorylated neurofilaments in the perikarya (Tortarolo et al., 2003). The accumulation of detergent-insoluble proteins and ubiquitinated intracellular inclusions are particularly evident at advanced stages of the disease (Cheroni et al., 2005). Reactive gliosis, which involves hypertrophy and the activation of astrocytes and the proliferation and activation of microglia, is detectable with the degeneration of motor neurons and becomes prominent when the cell loss is remarkable (Veglianese et al., 2006; Tortarolo et al., 2003). Hypertrophic astrocytes and reactive microglia are usually located around degenerating motor neurons.

For the present study female mice have been killed at 12, 18 and 23 weeks of age corresponding respectively to presymptomatic, early symptomatic and advanced stage of the motor dysfunction progression. Age-matched non transgenic littermates were used as controls.

2.2 IMMUNOHISTOCHEMISTRY

In this thesis fluorescence microscopy was used for a qualitative, but not quantitative, characterization of the distribution and expression of the AMPA receptor subunits and their trafficking proteins. Indeed, the quantification of fluorescence intensity is usually complicated by a variety of optical, biological, and physical factors. Another approach was considered to evaluate quantitatively these proteins, i.e. western blotting of the spinal cord described in section 2.3.

2.2.1 GENERAL PROCEDURE

Mice were anaesthetised with Equithesin (1% phenobarbitol / 4% (vol / vol) chloral hydrate, 30 µl /10 g, ip.) and transcardially perfused with 20 ml saline followed by 50 ml of sodium phosphate buffered 4% paraformaldehyde solution. Spinal cords were removed, post fixed in fixative for two hours, transferred to 20% sucrose solution in PBS overnight, then in 30% sucrose solution until they sank. Finally spinal cords were frozen in 2-methylbutane at -45°C and conserved at -80° until the experiments. Fixed and frozen lumbar spinal cords of transgenic and non transgenic mice were cut on a cryostat at -20°C to obtain coronal sections of 30 µm at the L2-L4 levels. The lumbar tract of the cord was chosen because the hind legs of these mice are affected earlier and more severely compared to the forelegs.

2.2.1.1 Indirect immunofluorescence

Free-floating sections were treated with a blocking solution composed of normal goat serum (NGS), Triton X-100 at appropriate concentration to permeabilize the membrane and PBS 0.01 M for 1 hour. Subsequently, the sections were washed and treated with a first antibody solution containing antibodies at appropriate concentration with NGS 10%, Triton at different concentration and PBS; all kept under mild agitation overnight at 4°C. Then the sections were treated with a fluorescence secondary antibody (1:500) directed to

different species (mouse, rabbit and rat) and conjugated to fluorochromes with different wavelengths (ALEXA 488, 546 and 647, Molecular Probes, Invitrogen) in NGS 1% and PBS for 1 hour and mounted with Fluorsave (Calbiochem).

2.2.1.2 Amplified immunofluorescence by Tyramide

Free-floating sections were first treated with H₂O₂ 1% in PBS 0.01 M for 10 minutes. Then, they were incubated in 10% NGS, Triton X-100 at appropriate concentration in PBS 0.01 M, for 1 hour and kept overnight at 4°C in the primary antibody solution at appropriate concentration containing 1% NGS and PBS. The following day, after three washes in PBS 0.01 M, sections were incubated with biotinylated secondary antibody (1:200, Vectastain kit, Vector Laboratories, Burlingame, CA, US) for 1 hour, washed with TNT (TBS 0.1 M; Triton 0.05%) and incubated with TNB (TBS 0.1 M, Blocking reagent 0.5%). Subsequently, the sections were again washed with TNT and treated with streptavidin (1:100, Perkin Elmer, NEN) in TNB for 30 minutes, washed with TNT and incubated for 10 minutes with Tyramide conjugated with Cy5 fluorochrome (1:500, Perkin Elmer, NEN) in amplification diluent provided by the kit. The amplification technique was utilized to detect faint signal or reveal two antibodies of the same specie on the same section, taking advantage of the sensibility of this technique for one of the antibodies.

2.2.1.3 Fluorogold

Fluorogold solution (FG) (a retrograde tracer that labels motor neurons following intramuscular injection, Fluorochrome) was injected in two site for both medial and lateral gastrocnemius muscle (5 µl/site with a 1% FG diluted in water) according to Zhang et al. (Zang da, Lopes et al. 2005). After 5 days, 5

control and transgenic mice at the presymptomatic stage of the disease were anesthetised with Equithesin (1% phenobarbital / 4% vol / vol/ chloral hydrate, 30 µl /10 g, ip.) and transcardially perfused with 20 ml saline followed by 50 ml of sodium phosphate buffered 4% paraformaldehyde solution. Spinal cords were removed, post fixed in fixative for two hours, transferred to 20% sucrose solution in PBS overnight, then in 30% sucrose solution until they sank. Finally spinal cords were frozen in 2-methylbutane at -45°C and conserved at -80° until the experiments. Fixed and frozen lumbar spinal cords of transgenic and non transgenic mice were cut on a cryostat at -20°C to obtain coronal sections of 30 µm at the L2-L4 levels.

Subsequently, on the sections FG was detected by immunofluorescence with the following procedure: free-floating sections were treated with a blocking solution composed of 10% NGS, 0.1% Triton X-100 and PBS 0.01 M for 1 hour. Then, the sections were washed and treated with a first antibody solution containing rabbit anti FG (1:1000) with NGS 10%, 0.1% Triton and PBS; all keep in shacking overnight at 4°C. After, the sections were treated with a fluorescence anti rabbit secondary antibody ALEXA 488 (1:500, Molecular probes, Invitrogen) in NGS 1% for 1 hour and mounted with fluorsave (Calbiochem).

2.2.2 IMMUNOFLUORESCENCE ANALYSIS OF GLUTAMATE AMPA RECEPTOR SUBUNITS

Immunohistochemical analysis was performed as described in section 2.2.1. Fixed lumbar spinal cords of SOD1G93A mice at 12, 18 and 23 weeks of age, corresponding to presymptomatic, symptomatic and end stage of the disease,

and non transgenic mice at the same age used as control (5 mice each group) were utilized.

The primary antibodies used were: rabbit polyclonal anti-GluR1, (1:100, Chemicon International, Harrow, UK), or mouse monoclonal anti-GluR2, (1:100, Chemicon International, Harrow, UK), or rabbit polyclonal anti-GluR2 serine 880 phosphorylated, (1:100, Upstate) or mouse monoclonal anti-GluR3 (1:100, Chemicon International, Harrow, UK) or rabbit polyclonal anti-GluR4 (1:100, Chemicon International, Harrow, UK), diluted in PBS 0.01M additioned with 10% NGS and 0.1% Triton X-100. Biotinylated anti mouse or anti rabbit secondary antibody was used. Subsequently, the signal was amplified with the Tyramide amplification procedure described in section 2.2.1.2. A second staining, at the presymptomatic stage of the disease, was carried out using a rabbit antibody directed to FG revealed by an anti rabbit secondary antibody conjugated to Alexa 488 as previously described in the section 2.2.1.3. At the symptomatic and end stage of the disease, a second staining using a rat monoclonal antibody directed to CD11b, a microglia marker (1:1000, produced by the Immunology laboratories in Mario Negri Institute), and a third staining using a mouse monoclonal antibody directed to GFAP, an astrocyte marker (1:1000, Chemicon International, Harrow, UK), were carried out by indirect immunofluorescence described in section 2.2.1.1. The secondary antibodies used were respectively an anti rat antibody conjugated to ALEXA 488 and anti mouse conjugated to ALEXA 546.

Controls for the specific signal analyzed was carried out with the procedure above mentioned, but omitting the first antibody. A lack of signal was obtained on the sections demonstrating specific signal for each staining.

Furthermore, a control of lack of cross signal using two primary antibodies of the same species on the same section was carried out. Because some of these antibodies are reised in a single species (GluR1,4 and FG), simultaneous immunoflourescent detection of multiple epitopes has been difficult. I have taken advantage of the high sensitivity of tyramide signal amplification for a protocol that permits simultaneous detection of two antibodies raised in the same species. One primary antibody was applied at a concentration below the detection limit of fluorescently labeled secondary antibodies, yet sufficient for detection with tyramide amplification (antibody ditected to GluR1 or 4). Whereas the antibody directed to FG have been detected by conventional immunofluorescence with a secondary antibody conjugated to ALEXA 488.

2.2.3 IMMUNOFLUORESCENCE ANALYSIS OF GLUTAMATE AMPA RECEPTOR TRAFFICKING PROTEINS

Immunohistochemical analysis was performed as described in section 2.2.1. Fixed lumbar spinal cords of SOD1G93A mice at 12, 18 and 23 weeks of age, corresponding to presymptomatic, symptomatic and end stage of the disease, and non transgenic mice at the same age used as control (5 mice each group) were utilized. The primary antibodies used were: rabbit polyclonal anti-ABP, (1:500, Chemicon International, Harrow, UK) or rabbit polyclonal anti-PICK1, (1:500, Abcam, Cambridge, UK) or rabbit polyclonal anti-NSF (1:500, Chemicon International), diluted in PBS 0.01M additioned with 10% NGS and 0.1% Triton X-100. Biotinylated anti rabbit secondary antibody was used. Subsequently, the signal was amplified with the Tyramide amplification procedure described in section 2.2.1.2.

A second staining at the presymptomatic stage of the disease was carried out with motor neurons marker (Fluorogold) as previously described in section 2.2.1.3. For the end stage of the disease, a second staining with a mouse monoclonal antibody directed to GFAP, an astrocytes marker (1:1000, Chemicon International, Harrow, UK), was carried out only for the slices stained for NSF by an indirect immunofluorescence described in section 2.2.1.1. The secondary antibody to reveal the astrocytes was an anti mouse conjugated to ALEXA 488.

Also in this case, such as for the AMPA receptors subunits, the controls for the specific signal analyzed were carried out with the procedure above mentioned, but omitting the first antibody. A lack of signal was obtained on the sections demonstrating specific signal for each staining. Furthermore, no cross signal in

the the study of colocalization of ABP, or PICK1 or NSF and FG (all antibodies made in rabbit) was obtained testing which is the dilution of the primary antibody directed to the AMPA receptor trafficking proteins detectable by tyramide, but below the threshold revealable by conventional secondary anti rabbit IgG conjugated with ALEXA 488 used to stain FG.

2.2.4 CONFOCAL ANALYSIS

The signals obtained were acquired with OLYMPUS Confocal System (FLUOVIEW 500) using three lasers: Ar with a 488 nm emission line, to acquire Alexa 488 signal, He-Ne green laser with a 546 emission line, to acquire Alexa 546 signal and He-Ne red laser with a 647 emission line, to acquire Cy5 signal. Analysis in double or triple staining was made by sequential scanning in order to avoid cross talk between the channels. Furthermore, a correct set up of pinholes was adopted for each wavelengths and magnification.

2.3 WESTERN BLOTTING

2.3.1 GENERAL PROCEDURE

Mice were killed by decapitation and the spinal cord and brain were rapidly removed and separated in lumbar, cervical and thoracic spinal cord. The lumbar spinal cord segment was longitudinally laid with his dorsal portion on a support of the OCT (mounting medium that solidify at -20°C), the ventral portion remaining superficial. The OCT included spinal cord segment was then placed in a cryostat with the ventral portion facing up. The ventral portion was then cut longitudinally in 50 µm thick sections until the appearance of the ependymal channel as limit between the ventral and dorsal portion. The sections from ventral or dorsal portion of the spinal cord were collected in an eppendorf tube and immediately frozen.

2.3.1.1 Western blot of total homogenate of the ventral and dorsal portion

Frozen ventral and dorsal spinal cords of transgenic and no transgenic mice were sonicated in boiling lysis buffer (20 mM Tris/HCl, pH 7.5, 10% SDS) and centrifuged at 13000 rpm for 3 minutes at 4°C.

2.3.1.2 Western blot of subcellular fractions of the ventral and dorsal portion

Sections of ventral and dorsal portion cut longitudinally in 50 μ m thick sections as above described of transgenic and no transgenic mice were washed with PBS 0.01 M to eliminate trace of OCT from the preparation.

Subsequently, ventral and dorsal portion of the lumbar spinal cord were homogenized in buffered sucrose (320 mM sucrose, 2 mM DTT, 1 mM EGTA, 1 mM EDTA, 4 mM HEPES-KOH, pH 7.4) supplemented with protease inhibitors in a glass-glass homogenizer. Cell debris and nuclei (P1) were removed by centrifugation for 10 min at 1.100g. Then, the postnuclear supernatant (S1) was centrifuged for 10 min at 9.200g, and the resulting pellet was resuspended in the buffered sucrose (320 mM sucrose, 2 mM DTT, 1 mM EGTA, 1 mM EDTA, 4 mM HEPES-KOH, pH 7.4). The resuspended pellet was further centrifuged for 15 min at 10.200g to obtain the washed synaptosomal fraction (P2). Supernatant from the medium-speed centrifugation was centrifuged for 2 h at 167.000g to obtain microsomal pellet (P3) and soluble protein fraction (S3). The washed P2 was lysed by addition of 10 volumes of water and the synaptosomal membrane-enriched fraction (LP1) was collected by centrifugation at 25.000g for 20 min (Lee, Valtschanoff et al. 2001).

2.3.1.3 Immunoblot

Ventral and dorsal total homogenates or their subcellular fractionation are subsequently characterized for the protein concentrations by using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Samples were then boiled for 3 minutes in loading buffer (100 mM Tris/HCl, pH 7.5, 15% α -mercaptoethanol, 4% SDS; 15% glycerol, 5 mM EGTA, 5 mM EDTA, 0.2% bromophenol blue) and run on polyacrylamide-SDS gel and transferred to nitrocellulose membrane (Scheicher and Schuell, Keene, NH, USA). Membranes were incubated in blocking buffer made of TBST (20 mM Tris/HCl, 150 mM NaCl, 0.05% Tween-20) with 5% skimmed milk for 1 hour at room temperature, followed by incubation overnight at 4°C with primary antibody diluted in TBST with 5% bovine serum albumin or skimmed milk. The blots were then washed three times in TBST and incubated with secondary antibody in TBST with 5% skimmed dry milk, for 1 hour at room temperature. Blots were then developed by the ECL technique (Amersham Biosciences) according to the manufacturer's instructions. Densitometric analysis of autoradiographic bands was done using a computer-assisted image analysis system (AIS 3.0, Imaging Research Inc.).

The quantitative evaluation of AMPA receptor subunits was expressed in relation to the level of actin as constitutive protein in the total homogenate, whereas a ratio between the optical density of the band specific for each subunit and that corresponding to PSD95 or GM130 from the same blot was carried out respectively for the LP1 and P3 fraction. A preliminary analysis was carried out demonstrating no changes in PSD95 and GM130 expression in transgenic mice compared to controls.

The level of purity of the two fractions was evaluated by the selective presence of PSD95 in post synaptic density contained in the LP1, but not in P3 fraction, and vice versa by the presence of GM130 exclusively in the P3, cytosolic fraction, but not in LP1 fraction.

As an internal control for each animal, I considered the ratio between the levels in ventral horn versus the dorsal horn in both transgenic and non transgenic mice.

Statistical analysis was performed using Mann-Whitney test (GraphPad Prism 2.0a for Power Macintosh, GraphPad Software Inc.).

2.3.2 WESTERN BLOT ANALYSIS OF GLUTAMATE AMPA RECEPTOR SUBUNITS IN SUBCELLULAR FRACTIONS OF LUMBAR SPINAL CORD

Western blot analysis was performed as described in section 2.3.1.2. Frozen spinal cords of SOD1G93A mice at presymptomatic stage of the disease and control mice at the same age (7 mice each group) were used. Thirty µg protein/lane of each sample were run on polyacrylamide-SDS gradient gel (7%) (Bio-Rad laboratorie, Hercules, CA, USA). The primary antibodies used were: polyclonal mouse anti-GluR1 (1:250, Chemicon International, Harrow, UK) or monoclonal mouse anti-GluR2 (1:500, Chemicon International, Harrow, UK) or monoclonal mouse anti-GluR3 (1:2000, Chemicon International, Harrow, UK) or polyclonal mouse anti-GluR4 (1:250, Chemicon International, Harrow, UK) or polyclonal mouse anti Post Synaptic Density protein 95 (PSD95) (1:500, made in Prof.Carlo Sala's laboratory, CNR, Milan) or monoclonal mouse anti-GM130, a protein that plays a role in the stacking of Golgi cisternae,

maintaining cis-Golgi structure and in vesicular transport, (1:500, BD Bioscience). Anti-mouse or anti rabbit IgG conjugated to horseradish peroxidase (1:2000, Sigma, Poole, UK) were used as secondary antibody. Blots were developed using ECL plus Western Blotting Detection System (Amersham Biosciences) according to the manufacturer's instructions.

2.3.3 WESTERN BLOT ANALYSIS OF GLUTAMATE AMPA RECEPTOR TRAFFICKING PROTEINS IN TOTAL HOMOGENATE OR SUBCELLULAR FRACTIONS OF LUMBAR SPINAL CORD

Western blot analysis was performed as described in section 2.3.1.1-2.3.1.2. Frozen spinal cords of SOD1G93A mice at presymptomatic stage of the disease and control mice at the same age (7 mice each group) were used. Eighty (total homogenate) or thirty (subcellular fractionation) µg protein/lane of each sample were run on polyacrylamide-SDS gradient gel (7%) (Bio-Rad laboratorie, Hercules, CA, USA). The primary antibodies used were: polyclonal rabbit anti-ABP (1:250, Chemicon International, Harrow, UK) or polyclonal rabbit anti-PICK1 (1:500, AbCAM, Cambridge, UK) or polyclonal rabbit anti-NSF (1:500, Chemicon International, Harrow, UK) or monoclonal mouse anti-Actin (1:250, Chemicon International, Harrow, UK) or polyclonal rabbit anti-PSD95 (1:500, Post Synaptic Density protein 95, made in Prof.Carlo Sala's laboratory, CNR, Milan) or monoclonal mouse anti-GM130 (1:500, a protein that plays roles in the stacking of Golgi cisternae, maintaining cis-Golgi structure and in vescicular transport., BD Bioscience). Anti-mouse or antirabbit IgG conjugated to horseradish peroxidase (1:2000, Sigma, Poole, UK) was used as secondary

antibody. Blots were developed using ECL plus Western Blotting Detection System (Amersham Biosciences) according to the manufacturer's instructions.

CHAPTER 3

Study of the expression of glutamate AMPA receptor subunits in the spinal cord of SOD1G93A mice during the progression of the disease

3.1 EXPRESSION AND DISTRIBUTION OF AMPA RECEPTOR SUBUNIT PROTEINS IN THE MOTOR NEURONS OF SOD1G93A MICE DURING THE PROGRESSION OF THE DISEASE EXAMINED BY IMMUNOFLUORESCENCE ANALYSIS

3.1.1 HYPOTHESIS AND AIM

Previous results from my laboratory have demonstrated by western blot and immunohistochemistry a decreased level of the GluR2 subunit in the ventral horn of SOD1G93A mice at the presymptomatic stage of the disease (Tortarolo et al., 2006).

The aim of this study is to further characterize the expression of the different AMPA receptor subunits selectively in the motor neurons of presymptomatic SOD1G93A mice. Motor neurons are specifically identified using fluorogold, a retrograde tracer injected at different sites of the gastrocnemius muscle. The distribution and expression of these subunits will be analyzed also during the progression of the disease. Moreover, I evaluate whether possible changes of post translational phosphorylation of the GluR2 subunit (GluR2-P), which is responsible for the removal of GluR2 from the postsynaptic membrane, may contribute to the development of the pathology.

3.1.2 RESULTS

3.1.2.1 Analysis of the expression and distribution of the AMPA receptor subunits in the motor neurons of SOD1G93A at the presymptomatic stage of the disease

Immunolabelling for AMPA receptor subunits was performed in the lumbar spinal cord of presymptomatic SOD1G93A mice compared to non transgenic mice.

Each subunit of AMPA receptors (GluR1-4) was analyzed in association with FG staining of motor neurons.

Fluorogold staining:

FG labelling showed a selective intense fluorescence in the motor neurons of the lamina IX of the ventral horn of the lumbar spinal cord, whereas no staining was observed in the dorsal horn (Fig 3, a). Motor neurons show a diffuse staining in perikarya and dendrites (Fig 3, b). The mechanism of transport of the FG from the neuromuscular junction to the cytosol of the motor neurons is not well characterized. However an active retrograde transport has been proposed. A limit of this FG could be the capacity to reach a different number of the motor neurons given that an altered axonal transport has been demonstrated in the G93A compared to CTR mouse. Hence, a preliminar study comparing the number of the motor neurons stained with FG for both CTR and G93A at the presymptomatic stage of the disease has been carried out demonstrating a

comparable capacity to stain the same number of the motor neurons at this stage of the disease analyzed.

GluR1 staining:

A diffuse staining for GluR1 subunits was evident in the spinal cord of control mice with an intense signal in the cells of the dorsal horn (substantia gelatinosa) and some scattered signal detectable in the ventral horn (Fig 3.1, A; a,b). At high magnification, the most intense staining was detected in both motor neuron perikarya and dendrites (Fig 3.1, A; c-d arrows). Some motor neurons of SOD1G93A mice stained with FG showed a massive vacuolization (Fig 3.1, A; g,h), however, no apparent changes in the expression of GluR1 subunits was evident in these cells comparing the intensity of colocalization signal of GluR1 and FG between control and transgenic mice (Fig 3.1, A; e,f,g,h). No differences was found also in the neuronal cells of the dorsal horn of the transgenic mice (Fig 3.1, A; a,b,e,f).

GluR2 staining:

Analysis of GluR2 showed a pattern of distribution similar to GluR1 in control mice with a staining in the neuronal cells of the ventral horn and in the dorsal horn. However, a much more intense signal of this subunit was specifically distributed in motor neuron cells stained with FG (Fig 3B, a-d). Unlike the GluR1, in transgenic mice at the presymptomatic stage of the disease, a

decreased staining was observed selectively in the cytoplasm of the motor neurons, but not in other neuronal cells. This appeared evident from a decreased colocalization with FG staining in SOD1G93A mice in respect to non transgenic controls (Fig 3.1, B; g,h arrow). It was also interesting to note that a decreased level of GluR2 was detected in motor neurons without apparent signs of degeneration (vacuolisation or morphological alteration) suggesting an early event in the motor neuron degeneration (Fig 3.1, B; g,h arrow). This further confirms that the decrease of this subunit occurs selectively in the motor neuron cells.

GluR3 staining:

GluR3 staining showed a pattern of distribution similar to GluR1 and 2 in the dorsal and ventral horns of the control mice with an intense signal in the FG positive motor neurons (Fig 3.2, A; a-d). Analyzing the transgenic mice no differences was revealed neither in the ventral horn (motor neurons did not show changes of colocalization intensity between GluR3 and FG) nor in the dorsal horn (Fig 3.2, A; e-h).

GluR4 staining:

GluR4 showed a staining throughout the grey matter and a clear staining in the perikarya of the motor neurons in ventral horn of control mice stained with FG (Fig 3.2, B; a-d). Also in this case, as for GluR3 and GluR1, evaluating the transgenic mice no differences were detected at this stage of the disease in the ventral (no changes of colocalization intensity between GluR4 and FG) and in the dorsal horn compared to controls (Fig 3.2, B; e-h).

3.1.2.2 Analysis of GluR2 phosphorylation in the motor neurons of SOD1G93A at the presymptomatic stage of the disease

GluR2-P staining:

In order to study whether a post translational event could influence the trafficking and the level of GluR2 in the motor neurons, an immunofluorescence analysis with an antibody directed to the phosphorylated form of GluR2 at the serine site 880 was carried out in control and transgenic mice. A diffuse staining was found in the control mice mostly localized in the ventral horn and in the substantia gelatinosa of the dorsal horn (Fig 3.3; b). In the ventral horn a strong signal was found in the motor neurons with an higher staining localized in proximity of the membrane (Fig 3.3; d,e) suggesting a preferential localization of phosphorylated GluR2 in the postsynaptic membrane. Analyzing the transgenic mice at the presymptomatic stage of the disease, no significant changes was observed compared to control mice (Fig 3.3; b,c), although a reduced staining in few morphologically altered motor neurons was present (Fig 3.3, f, arrow).

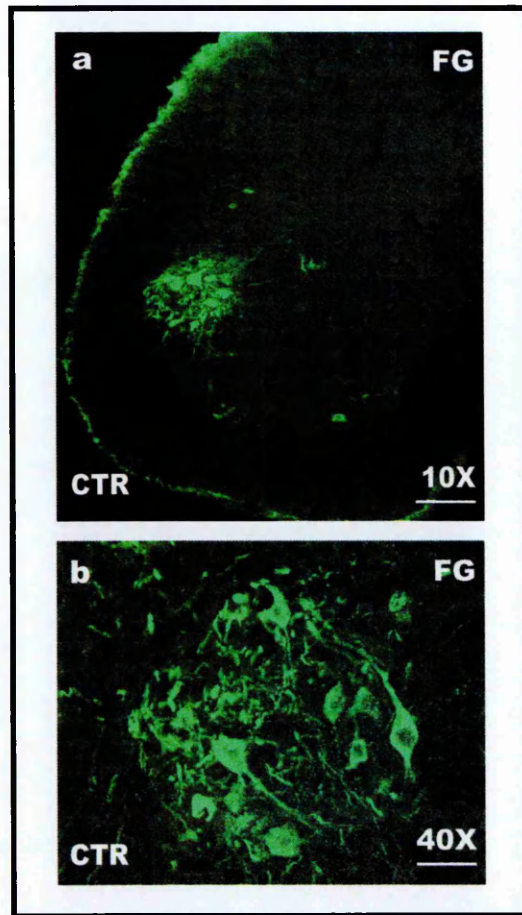


Fig 3: FG immunofluorescence in lumbar spinal cord of control mice

In control mice a selective Fluorogold (FG) immunoreactivity (green) is localized in the ventral horn of the grey matter with a marked staining in a pool of ventral horn motor neurons corresponding to the lamina IX (a). At high magnification FG immunostaining is distributed in the perikarya and dendrites of the motor neurons (b). Scale bars: (a = 200 μ m); (b= 50 μ m).

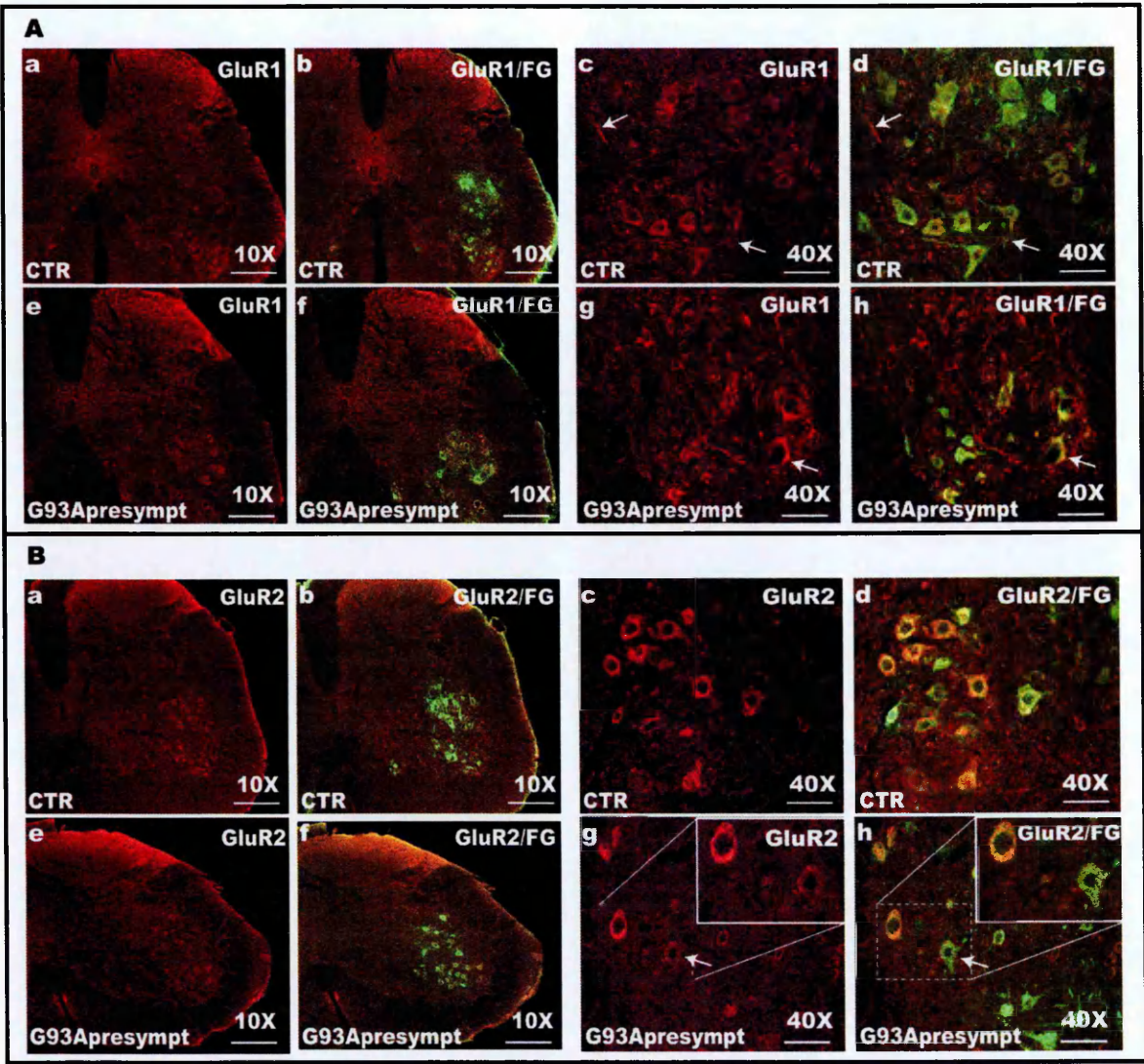


Fig 3.1: GluR1 and GluR2 immunofluorescence in lumbar spinal cord of SOD1G93A mice at the presymptomatic stage of the disease

In control mice a widespread GluR1 and GluR2 immunoreactivity (red) is localized throughout the whole grey matter with a marked staining in the dorsal horn and in motor neurons of ventral horn (A,B; a,c;). In the motor neurons, stained by Fluorogold (FG, green), GluR1 immunostaining is distributed in cell body and neurites (A; c, arrows). No significant changes in GluR1 immunostaining are observed in the dorsal horn of SOD1G93A mice (A; a,e), and in ventral horn (A; a,c,e,g), whereas, decreased

labelling is revealed in the ventral region for GluR2 (B; c,g,miniature). In particular, we could observe a decrease of GluR2 immunostaining in the perikarya of some the motor neurons (B; g, arrow). Scale bars: (A,B; a,b,e,f = 200 μ m); (A,B; c,d,g,h= 50 μ m).

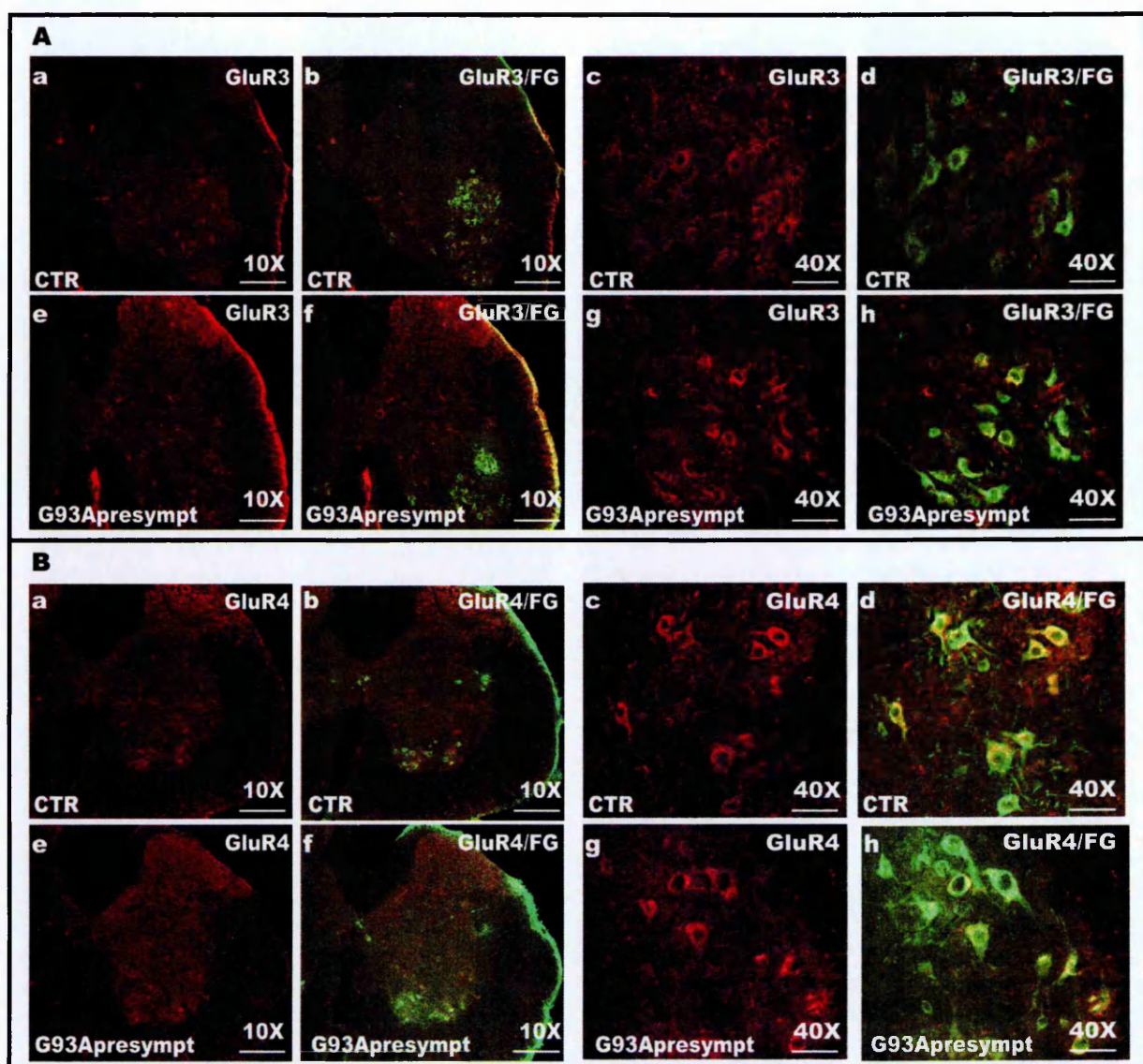


Fig 3.2: GluR3 and GluR4 immunofluorescence in lumbar spinal cord of SOD1G93A mice at the presymptomatic stage of the disease

In control mice, a widespread GluR3 and GluR4 immunoreactivity (red) is localized throughout the whole grey matter with a marked staining in the dorsal horn and in motor neurons of ventral horn (A,B; a,c). In the motor neurons, stained by Fluorogold (FG, green), GluR3 and GluR4 immunostaining is distributed in the perikarya and neurites (A,B; c). No significant changes in GluR3 and GluR4 immunostaining are

observed in the ventral and dorsal horn of SOD1G93A mice (A,B; a-h). Scale bars: (A,B; a,b,e,f = 200 μ m); (A,B; c,d,g,h= 50 μ m).

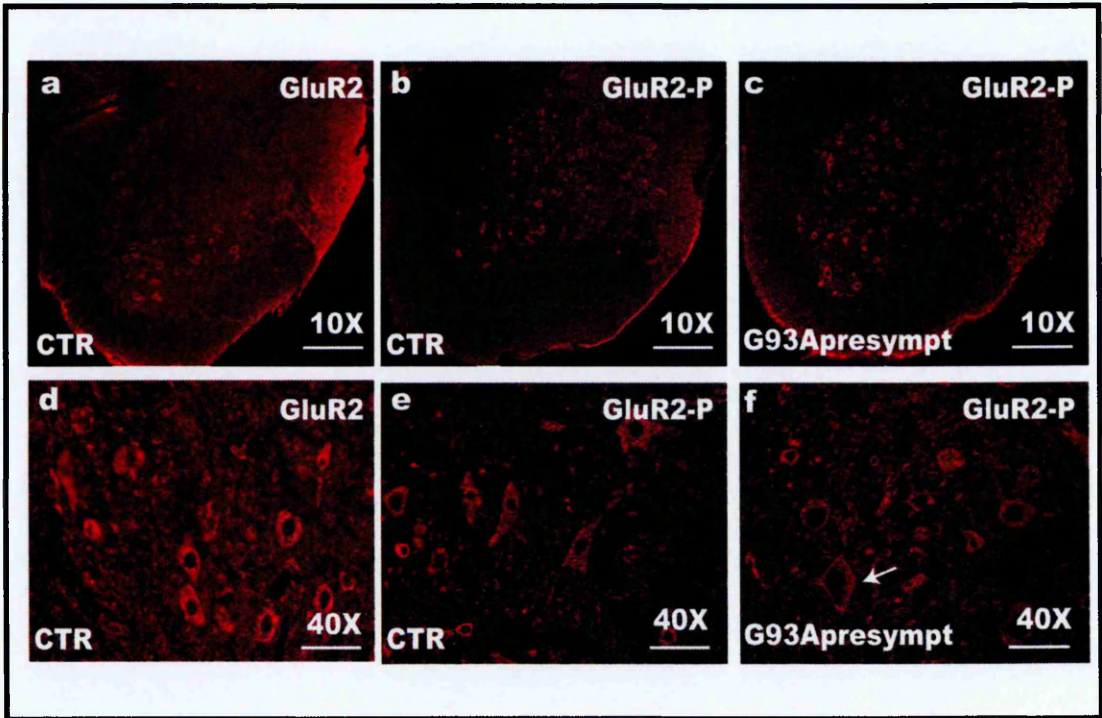


Fig 3.3: GluR2-P immunofluorescence in lumbar spinal cord of SOD1G93A mice at the presymptomatic stage of the disease

A diffuse immunoreactivity of phosphorylated GluR2 at the serine site 880 (GluR2-P, red) is localized throughout the whole grey matter with a marked staining in the dorsal horn and in motor neurons of ventral horn of control mice (b,e). In the motor neurons a faint signal is present in the perikarya, whereas a more intense staining is localized in proximity of the membrane (e) compared to GluR2 (d). No significant changes in GluR2-P immunostaining are observed in the ventral horn (e,f) and dorsal horn (b,c) of SOD1G93A mice compared to control mice. Interestingly some vacuolized motor neurons preserve a membrane staining of the GluR2-P (f, arrow). Scale bars: (a,b,c = 200 μ m); (e,f,d= 50 μ m).

3.1.2.3 Analysis of the distribution and the expression of the AMPA receptor subunits and GluR2-P during the progression of the disease, at symptomatic and final stage of the pathology

Subsequently, the distribution and expression of GluR1, 2, 3, 4 and GluR2-P were evaluated during the progression of the disease at symptomatic and end stage.

In order to characterize the non-neuronal staining, a study of colocalization in triple staining with markers for both the microglia cells (CD11b, green) and astrocytes (GFAP, blue) was performed as described above in section 2.2.1.1.

GluR1 staining:

A scattered signal particularly evident in the ventral horn at the symptomatic stage (Fig 3.4, b) that becomes more diffuse in the whole grey matter at the final stage (Fig 3.4, c) was detected in the transgenic compared to control mice (Fig 3.4, a). A scattered GluR1 signal was observed around the motor neurons at the symptomatic stage (Fig 3.5, b) and becomes more intense and diffuse at the endstage (Fig 3.5, c). The survived motor neuron cells did not show any differences at both the stages examined compared to control (Fig 3.5, a). A partial colocalization at symptomatic stage (Fig 3.6, B; f,g/3.10, A; b,c) and a more intense and diffuse colabeling at the endstage of the disease (Fig 3.6, B; j,k/3.10, B; b,c) was found with GFAP immunostaining which recognized hypertrophic astrocytes. No colocalization with the marker for activated microglial cells has been reported at all the stages of the disease examined (Fig 3.6, B; f,h,j,l).

GluR2 staining:

Unlike for the presymptomatic mice that showed a decrease staining in few motor neurons, there were no clear differences in the GluR2 immunostaining in survived motor neurons at the symptomatic (Fig 3.5, e) and end stage (Fig 3.5, f) of the disease compared to control mice (Fig 3.5, d). Instead, with the progression of the disease an intense scattered signal was present around the motor neurons. Analyzing thoroughly such scattered signal in experiments of triple staining with markers for both microglia and astrocytes, a clear colocalization between GluR2 and marker of microglia has been detected at the symptomatic stage (Fig 3.7, B; f,h/3.10, A; e,f) and even more at the end stage the disease (Fig 3.7, B; j,l/3.10, B; e,f). No colocalization was found with GFAP labelled astrocytes (Fig 3.7, B; g,k,c).

GluR3 staining:

Also the GluR3 immunostaining appeared intense and scattered around the motor neurons at the symptomatic and end stage (Fig 3.4-3.5, g, h, i). Examining the colocalization of GluR3 with marker for astrocytes (GFAP) and microglia (CD11b), we observed an overlapping staining of scattered signal with CD11b, but not with GFAP at the symptomatic (Fig 3.8, B; f,g,h/3.10, A; h,i) and even more at the end stage of the disease (Fig 3.8, B; j,k,l/3.10, B; h,i). Survived motor neurons of SOD1G93A mice at both stages showed no difference in the GluR3 immunostaining in respect to controls (Fig 3.5, g, h, i).

GluR4 staining:

No changes of GluR4 staining in the reactive glial cells has been documented in the transgenic mice during the progression of the disease at the symptomatic (Fig 3.9, B; e-h) and end stage (Fig 3.9, B; i-l) of the disease, such as demonstrated by lack of colocalization between GluR4 and the markers for astrocytes (GFAP) and reactive microglial cells (CD11b). Furthermore, the staining of GluR4 in the survived motor neurons of the transgenic mice appeared not changed compared to control motor neurons (Fig 3.5; j-l).

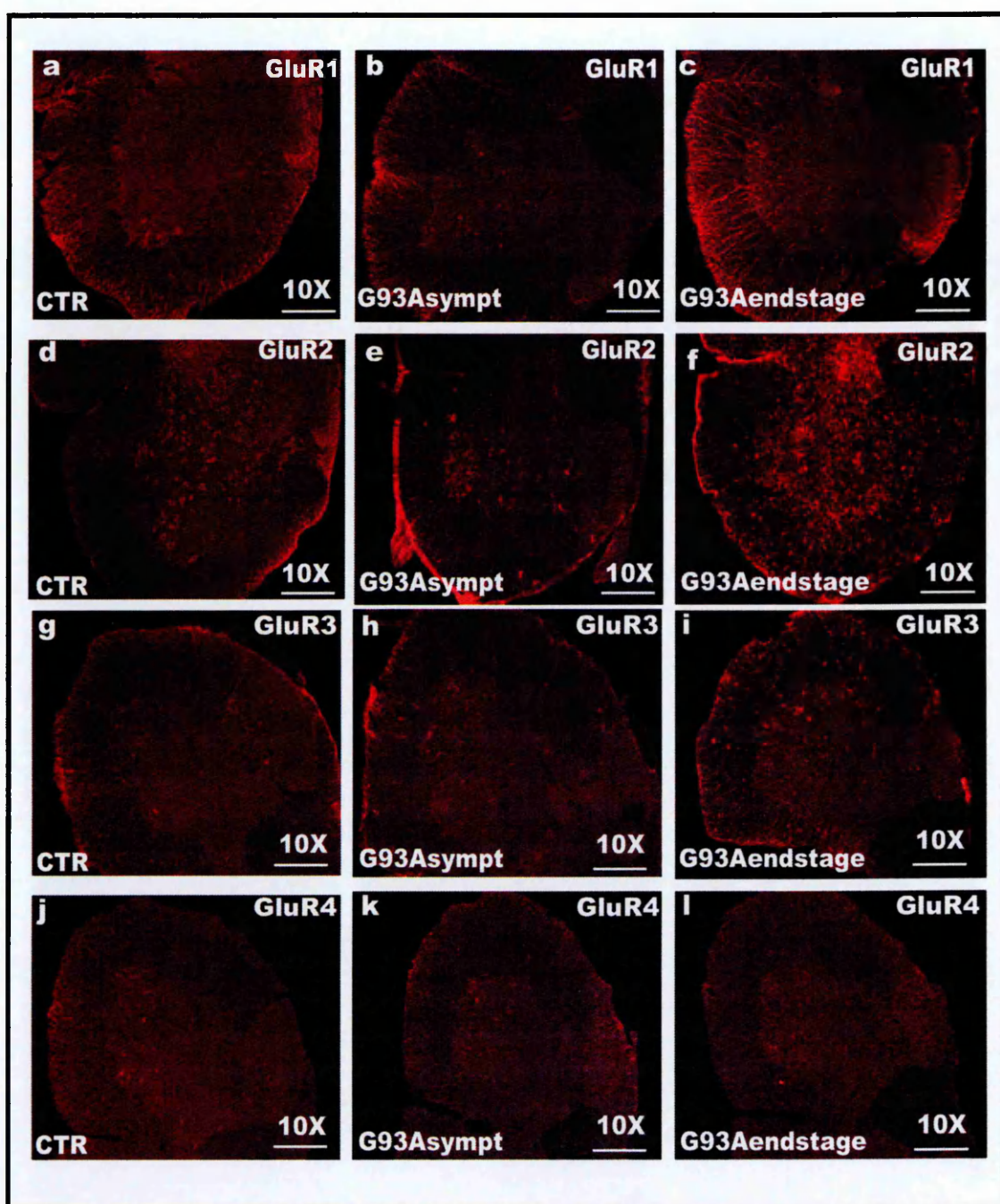


Fig 3.4: GluRs immunofluorescence at the symptomatic and end stage of the disease in lumbar spinal cord of SOD1G93A mice (Low magnification)

A scattered immunoreactivity (red) is revealed in the ventral horn of transgenic mice (b,e,h) at the symptomatic stage for GluR1,2 and 3 compared to control mice (a,d,g). At the endstage these diffuse staining become more pronounced and diffuse in the whole

grey matter (c,f,i). No differences between control (j) and transgenic is detected at both symptomatic and end stages of the disease for GluR4 (k,l). Scale bars: (a-l = 200 μ m).

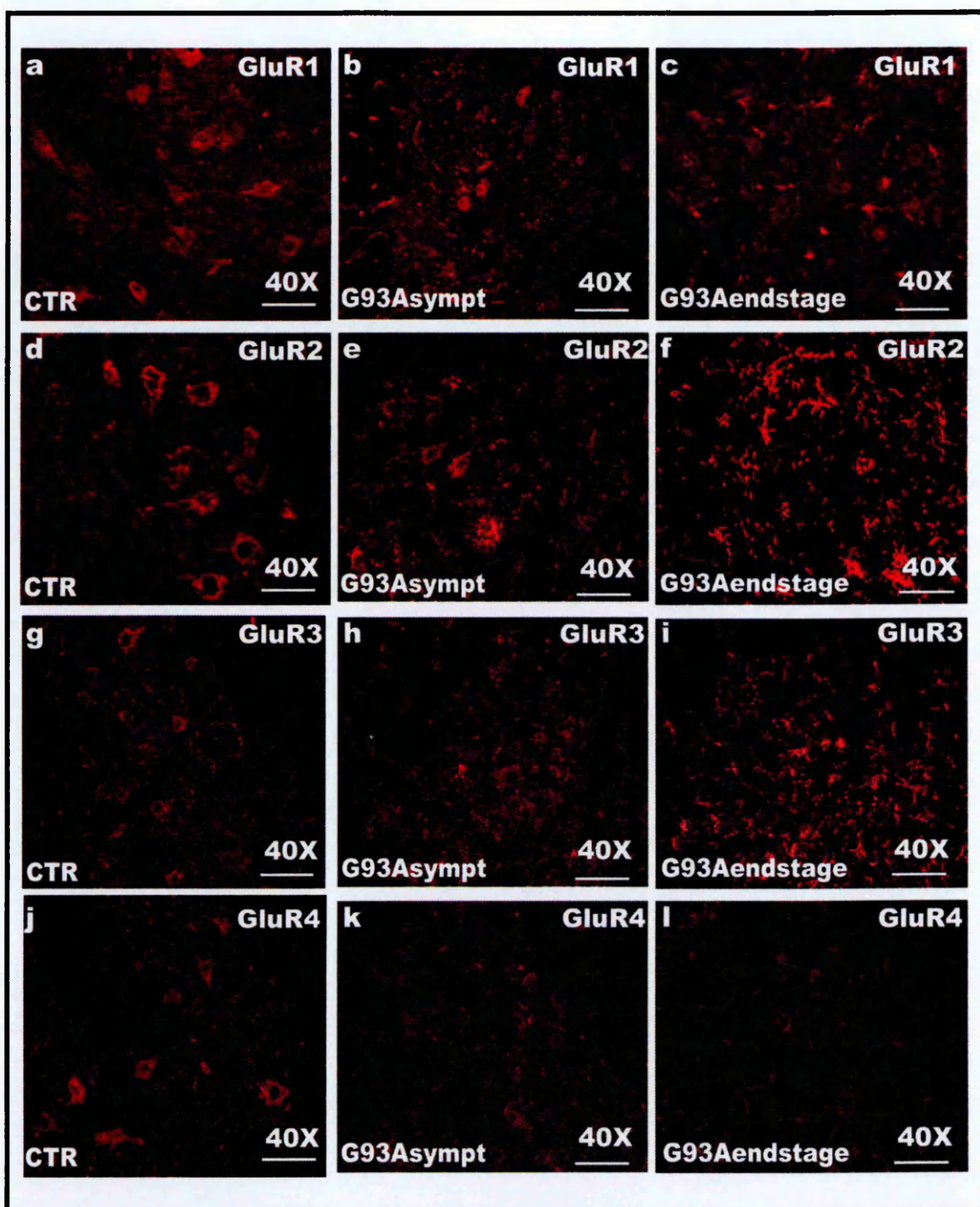


Fig 3.5: GluRs immunofluorescence at the symptomatic and end stage of the disease in lumbar spinal cord of SOD1G93A mice (High magnification)

A scattered staining is reported around the motor neurons for GluR1,2 and 3 (b,e,h), but not for GluR4 (k). Whereas, no clear differences in intensity or distribution of the signal is present comparing motor neurons in control (a,d,g,j) and transgenic mice (b,e,h,k) for GluR1,2,3 and 4 at the symptomatic stage. At the endstage of the disease, a

reduced amount of motor neurons is present with a predominant scattered staining for GluR1,2 and 3 around them (c,f,i), whereas GluR4 (l) in transgenic mice do not show difference with the control mice (j). Scale bars: (a-l = 200 μ m).

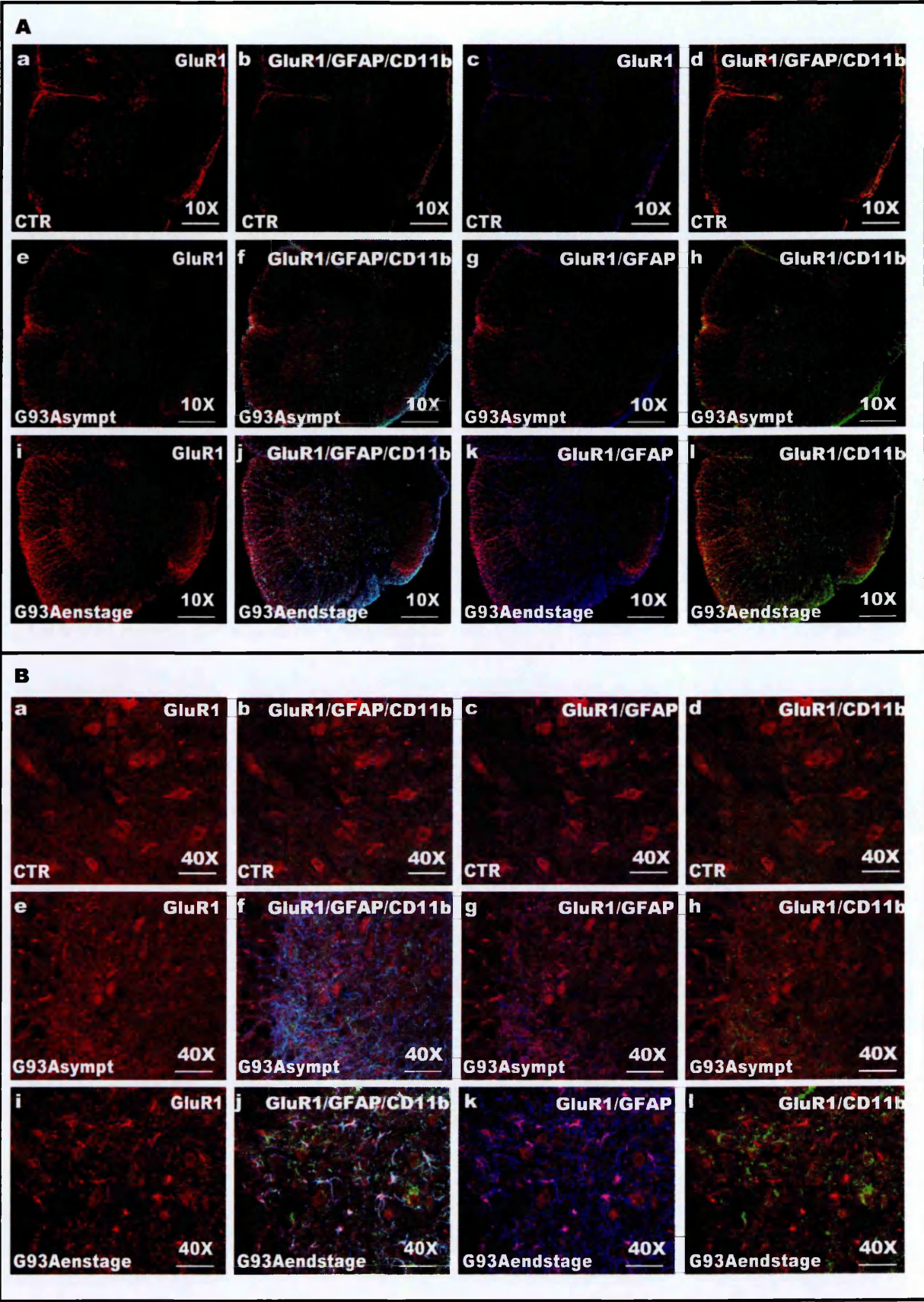


Fig 3.6: Study of colocalization of GluR1 with glial cells at the symptomatic and end stage of the disease in lumbar spinal cord of SOD1G93A mice

During the progression of the disease an increase gliosis, represented by an augmented immunoreactivity for both markers for astrocytes (GFAP,blue) and microglial cells (CD11b,green), is reported mostly in the ventral horn at the symptomatic stage (A; f,g,h). At the endstage hypertrophic astrocytes and reactive microglia diffuse in the whole spinal cord (A; j,k,l). Analyzing GluR1 signal (red), a scattered immunostaining is reported in the ventral horn at the symptomatic stage diffusing in the whole grey matter at the end stage (A; e,i). At high magnification, a study of colocalization shows an increased staining of GluR1 exclusively in the hypertrophic astrocytes (violet) (B; f,j,g,k), but not in reactive microglial cells at symptomatic stage. A colocalization staining of GluR1 with the hypertrophic astrocytes is much more represented at the end stage of the disease (B; f,j,h,l). Scale bars: (A; a,l = 200 μ m); (B; a,l= 50 μ m)

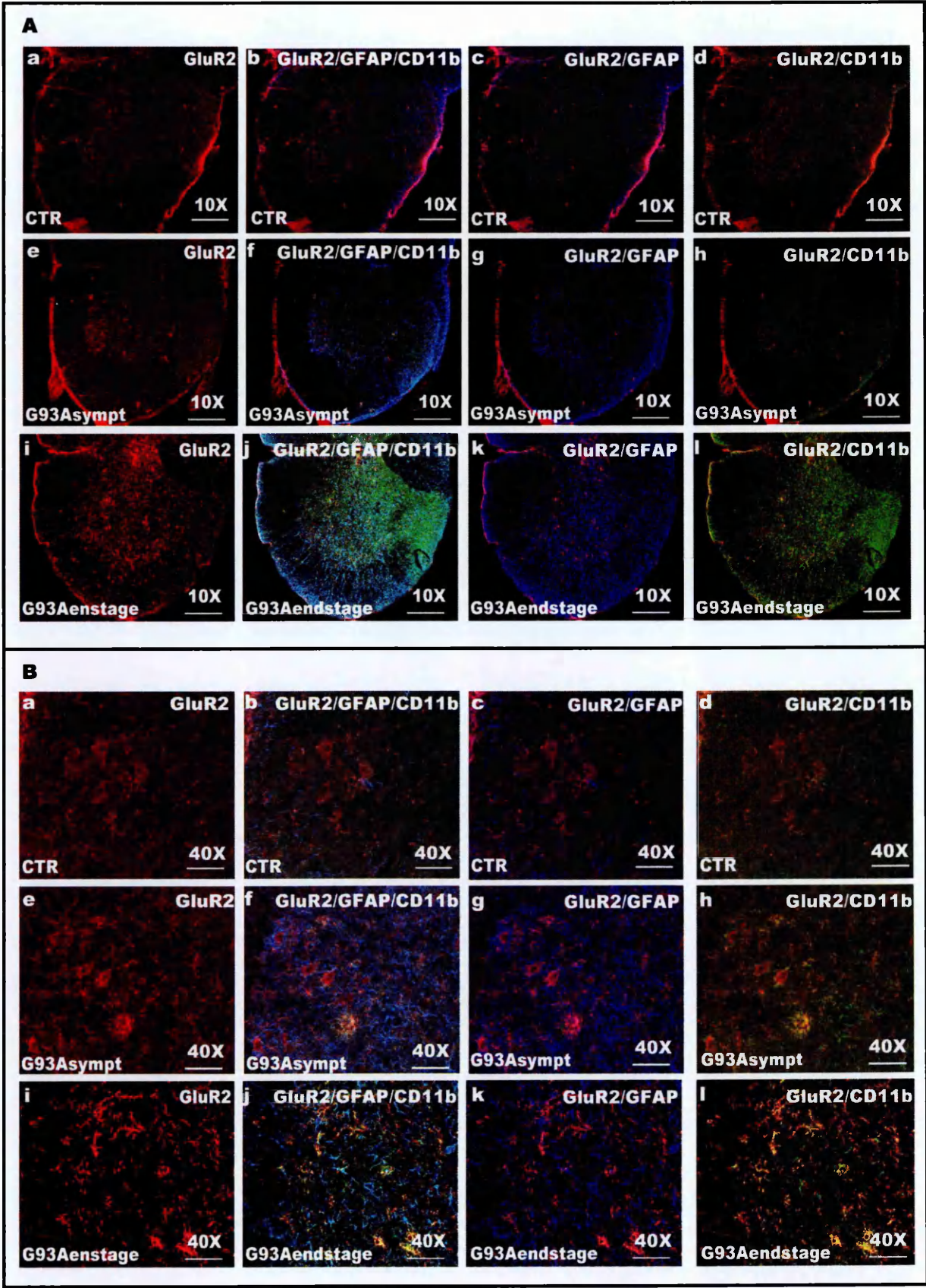


Fig 3.7: Study of colocalization of GluR2 with glial cells at the symptomatic and end stage of the disease in lumbar spinal cord of SOD1G93A mice

An increased diffuse immunoreactivity of GluR2 (red) is localized in the ventral horn of the transgenic mice at the presymptomatic stage (A, e) compared to control mice (A, a). This signal becomes more diffuse and intense at the end stage of the disease throughout the whole grey matter (A,i).

Analyzing at high magnification the colocalization of GluR2 with the glial cells, a colabeling exclusively with the microglia (CD11b, green) is present at the symptomatic stage of the disease (B, f,h) that becomes more intense and diffuse at the end stage (B; j,l), whereas GluR2 is not present in astrocytes (GFAP, blue) (B; g,k) compared to control (B; c). Scale bars: (A; a,l = 200 μ m); (B; a,l= 50 μ m)

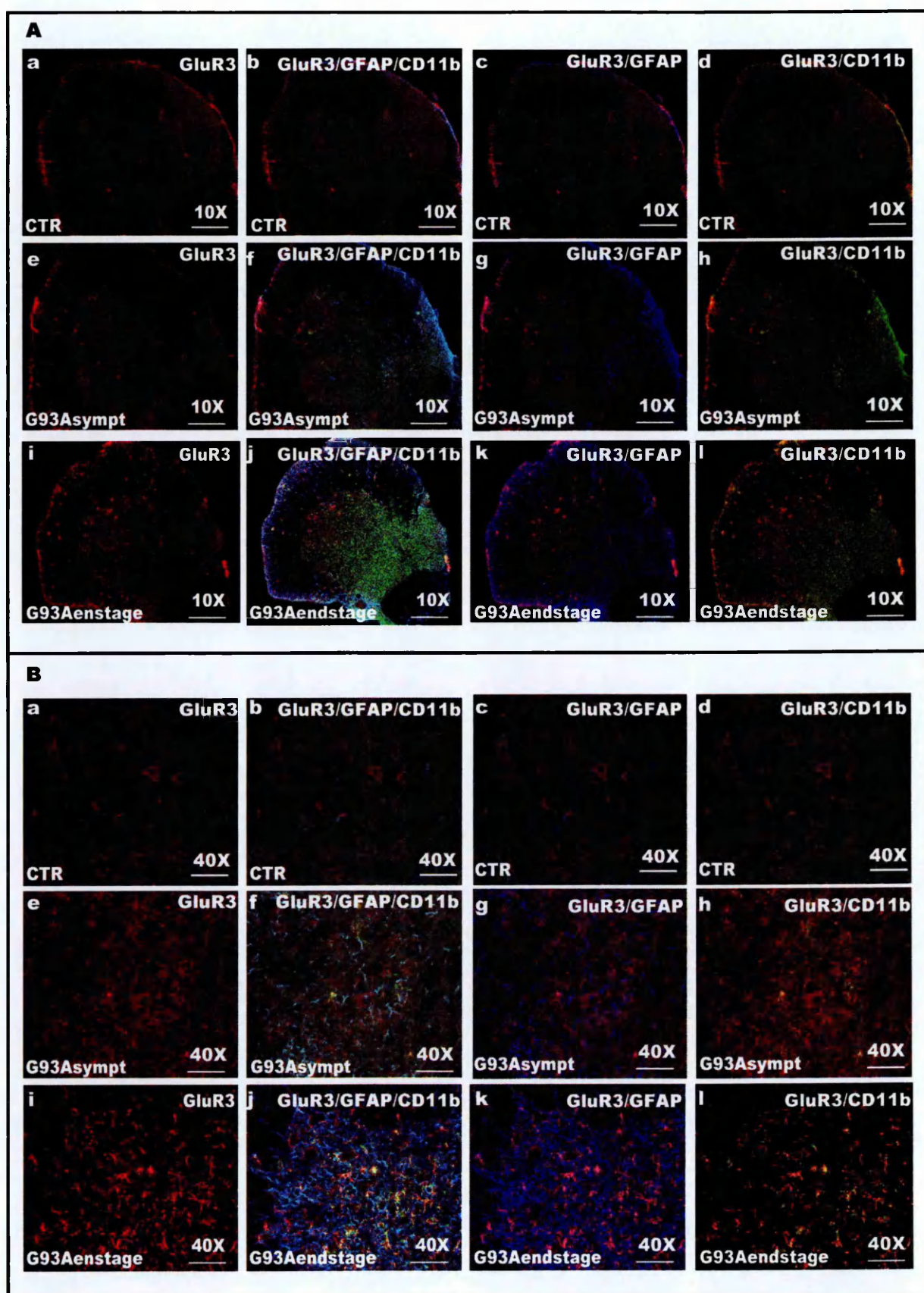


Fig 3.8: Study of colocalization of GluR3 with glial cells at the symptomatic and end stage of the disease in lumbar spinal cord of SOD1G93A mice

An increased diffuse staining of GluR3 (red) is localized in the ventral horn of the transgenic mice at the symptomatic stage (A, e), that becomes more intense and diffuse at the end stage of the disease (A, i). Analyzing at high magnification the colocalization of GluR3 with the glial cells, a colabeling exclusively with the microglial cells (CD11b, green) is present at both the symptomatic (B; f,h) and end stage of the disease (B; j,l), whereas no colocalization is present with astrocytes (GFAP, blue) at all the stages examined (B;g,k) compared to control (B,c). Scale bars: (A; a,l = 200 μ m); (B; a,l= 50 μ m)

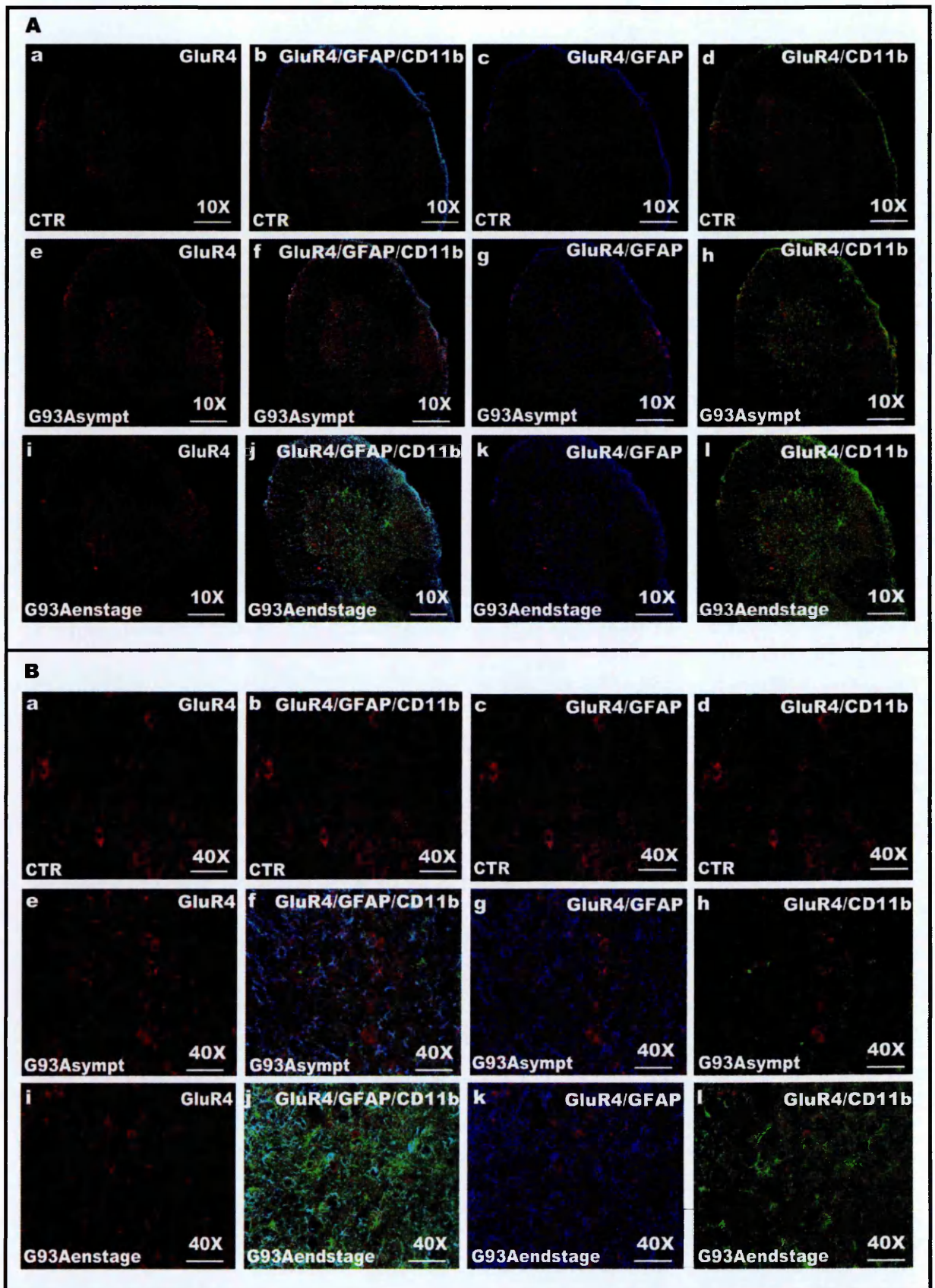


Fig 3.9: Study of colocalization of GluR4 with glial cells in lumbar spinal cord of SOD1G93A mice at the symptomatic and end stage of the disease

A GluR4 staining is distributed in the ventral and dorsal horn of the spinal cord of control mice (A, a-d). At high magnification a staining in the perikarya of the motor neurons is revealed (B, a-d). Analyzing the transgenic mice no differences are observed in survived motor neurons and glial cells at both symptomatic (A,B, e-h) and endstage (A,B, i-l) of the disease.

Scale bars: (A; a,l = 200 μ m); (B; a,l= 50 μ m)

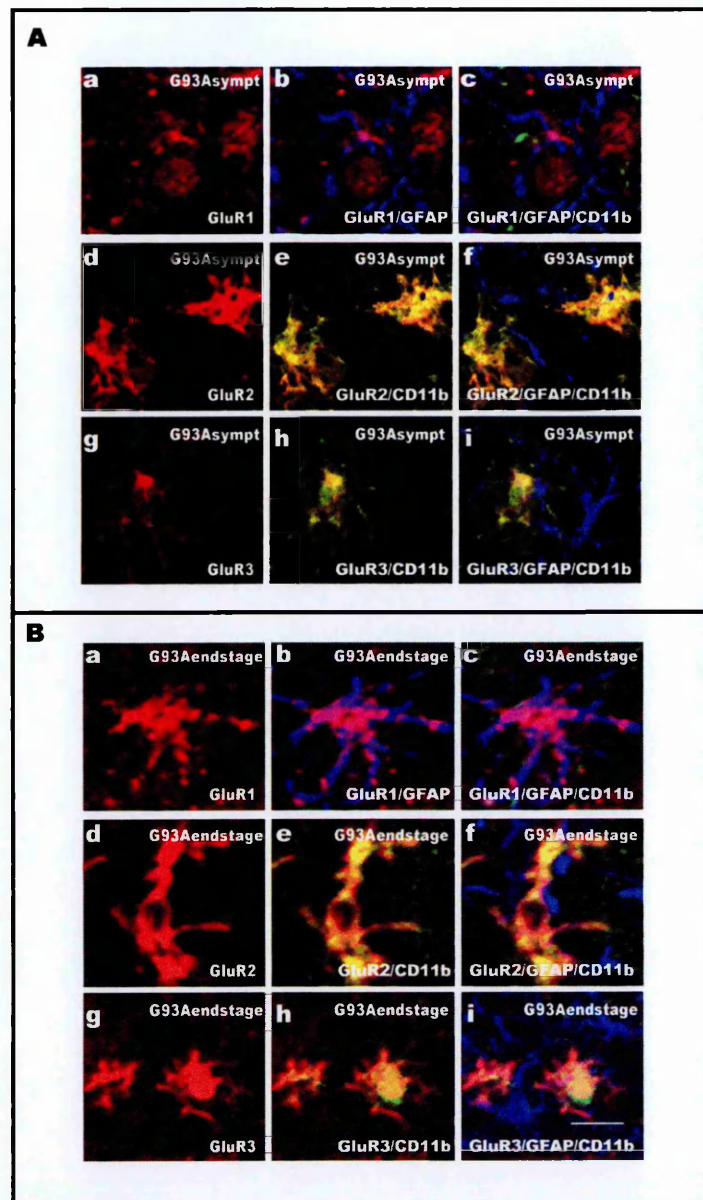


Fig 3.10: Study of colocalization of GluRs with glial cells in lumbar spinal cord of SOD1G93A mice at the symptomatic and end stage of the disease (high magnification)

High magnification of the colocalization of GluR1 and 2,3 respectively with hypertrophic astrocytes (GFAP, blue) and the microglial cells (CD11b, green) at both the symptomatic (A; b,c,e,f,h,i) and end stage of the disease (B; b,c,e,f,h,i). Scale bars: (A,B, a-i = 20 μ m)

3.2 STUDY OF SUBCELLULAR DISTRIBUTION OF GLUTAMATE AMPA RECEPTOR SUBUNITS IN THE SPINAL CORD OF SOD1G93A MICE

3.2.1 HYPOTHESIS AND AIM

It was suggested that alterations in the postsynaptic membrane stoichiometry of AMPA receptor subunits lead to modification in synaptic efficacy of glutamatergic transmission which may be potentially neurotoxic. In order to define if this phenomenon may contribute to the motor neuron death in ALS, in this part of the work I have examined the composition of AMPA receptor subunits in the postsynaptic membrane, in respect to the cytosol where the receptors are stored and ready to be carried to the postsynaptic membrane or addressed to the degradation. The analysis was carried out in lumbar spinal cord of SOD1G93A at the presymptomatic stage of the disease compared with non transgenic littermates.

3.2.2 RESULTS

In order to evaluate an early alteration of the subcellular distribution of the AMPA receptor subunits, and the phosphorylated form of GluR2. This study investigated only the presymptomatic stage of the disease of the transgenic mice compared to age matched control mice. A further reason to evaluate only the presymptomatic stage is the attempt to relieve a possible alteration of these subunits selectively in the motor neurons. Indeed, the results reported in section 3.1 of this thesis have shown an alteration of these subunits also in the glial cells during the progression of the disease affecting quantitative estimation of the AMPA subunits in the motor neurons at other stages.

I have examined the ventral and the dorsal horn of the lumbar spinal cord separately as described above in section 2.3.1.2-2.3.2. Two fractions were analyzed, a cytosol fraction (P3) and a synaptosomal membrane enriched fraction (LP1) from the ventral and dorsal portion.

GluR1 subunit:

The amount of GluR1 AMPA subunit protein expressed in the ventral and dorsal spinal cord of SOD1G93A mice was tested at the presymptomatic stage of the disease. Analyzing the two fractions, only a detectable band of ~100 KDa for GluR1 in the LP1 has been revealed (Fig 3.11A), whereas P3 has shown no detectable signal.

No significant differences in the level of GluR1 in LP1 fraction were observed comparing the controls to transgenic mice (Fig 3.11A).

GluR2 subunit:

An intense band of ~100 KDa for GluR2 has been also detected in P3 fraction suggesting a redistribution of this subunit between the cytoplasm and the postsynaptic membrane (Fig 3.11C). Interestingly, whereas a quantitative determination of the optical density of the band of GluR2 in LP1 fraction did not reveal any significant change when comparing controls to transgenic mice, a significant decrease was detected in P3 fractions of transgenic animals (Fig 3.11C).

GluR2-P subunit:

With the aim to define whether events of phosphorylation could alter expression or trafficking of the GluR2 subunit in postsynapse, the phosphorylated status of the GluR2 in LP1 fraction was analyzed. The result obtained has shown no changes between the quantitative evaluation of the specific bands of ~100 KDa obtained in controls and transgenic mice (Fig 3.11D).

GluR3 subunit:

An intense band of ~100 KDa was seen also for GluR3 subunit in both the fractions considered. However, contrary to the decreased expression of GluR2 in the P3 fraction, Glu3 did not show any changes of band intensity between controls and transgenic mice in both analyzed fractions (Fig 3.11E).

GluR4 subunit:

As GluR3, an intense band of ~100 KDa was seen for GluR4 subunit in both LP1 and P3. Also in this case, no significant change was detected when comparing control to transgenic mice (Fig 3.11F).

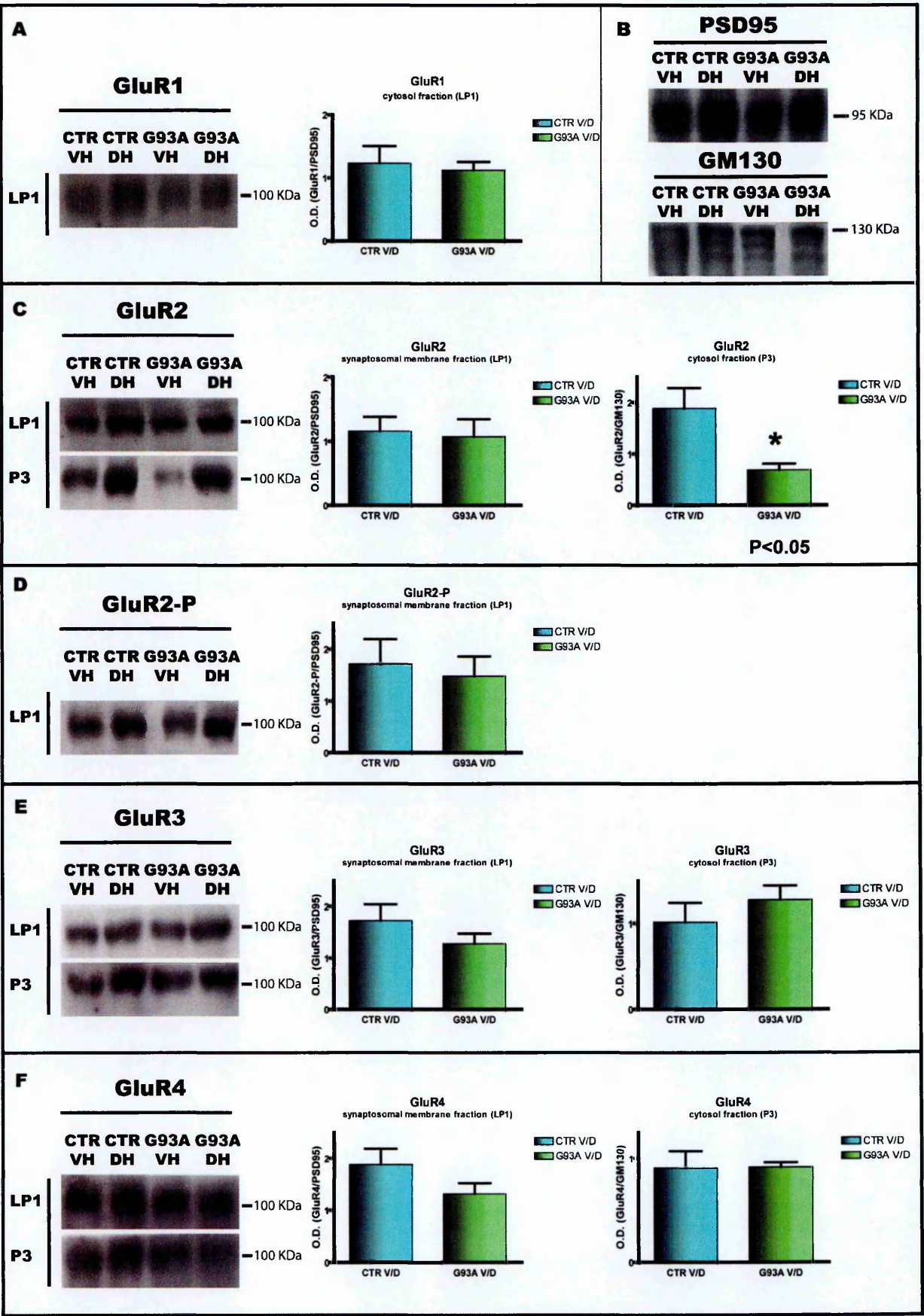


Fig 3.11: Western blot analysis of the expression of AMPA receptor subunits in subcellular fractionation of ventral and dorsal spinal cord of SOD1G93A mice

Results show quantitative evaluation of GluR1, GluR2, GluR2-P, GluR3 and GluR4 (A,C-F) in the cytosol fraction (P3) and synaptosomal membrane enriched fraction (LP1) obtained from ventral (VH) and dorsal (DH) portion of lumbar spinal cord of SOD1G93A mice at the presymptomatic stage, and their non transgenic littermate. The protein levels are expressed as ratio between band intensity and the optical density of each GluRs band compared to that PSD95 (B) in the same blot for the LP1 fraction, whereas as ratio with GM130 (B) for the P3 fraction; this to normalize a possible different loading. A ratio between the VH and DH portion for each fractions was carried out. No evident differences in GluR1, GluR2-P, GluR3 and GluR4 band intensity were observed in both the fractions LP1 and P3 in SOD1G93A mice at presymptomatic stage of the disease when compared to controls at the same age (A,D-F). Whereas, a significant decrease in GluR2 band intensity was only found in the P3 fraction of transgenic mice compared to control (C). Each column shows the mean \pm S.E.M (n=7). Data analysed by Mann Whitney test.

3.3 DISCUSSION

Several data suggest that AMPA receptors can be involved in the onset and the progression of the disease in SOD1 mutant mice. Alterations of the molecular features and composition of AMPA receptors may result in aberrant glutamatergic transmission and increased calcium entry, which in turn can lead to cell death. It is still unclear, however, how they can be regulated to determine the selective vulnerability of spinal motor neurons.

In this part of the project I have demonstrated that the sciatic nerve pool of motor neurons in lumbar spinal cord selectively labelled by FG injected in the hind limb muscles of presymptomatic SOD1G93A mice show lower levels of the GluR2 AMPA subunit in respect to those of control mice. In particular here, I demonstrated that the reduction in GluR2 is specific for motor neurons because of its colocalization with FG, which label selectively the sciatic nerve pool of motor neurons, was remarkably reduced.

In line with these data, we also found a decrease of GluR2 by western blot, but only at the level of the cytosol. The fact that these alterations were observed in mice at the presymptomatic stage of the disease, suggests that this event may have a causative role in triggering the pathology in these mice.

Previous data from our lab showed no changes of GluR2 mRNA in the motor neurons of presymptomatic SOD1G93A mice, demonstrating that reduced level of GluR2 protein is not related to a decreased synthesis (Tortarolo, Grignaschi et al. 2006). This indicates that a post translational event can lead to decrease of GluR2 protein expression in the lumbar motor neurons of SOD1G93A mice.

AMPA receptors cycle rapidly between intracellular and plasma membranes and the amount of internalized receptor are strongly affected by the rate of recycling to the surface as well as by the rate of endocytosis (Ehlers 2000; Lee, Simonetta et al. 2004). It was demonstrated that a rapid loss of synaptic AMPA receptors occurred following synaptic stimulation with subsequent induction of long-term depression (LTD) (Carroll, Lissin et al. 1999) or with exogenous application of glutamate (Lissin, Carroll et al. 1999). Furthermore, experiments carried out in neuronal culture have demonstrated that AMPA receptors activation causes a Ca^{2+} -independent internalization of AMPARs, which are then targeted to endosomes and degraded by lysosomes (Ehlers 2000). After endocytosis, AMPA receptors undergo endosomal sorting like any other internalized membrane protein, then they can be recycled back to the surface membrane or degraded in lysosomes (Ehlers 2000; Lin, Ju et al. 2000; Gruenberg 2001). Other studies have demonstrated that others pathways of degradation such as the proteasome were not involved in the reduction of the AMPA receptor protein level (Lee, Simonetta et al. 2004). A depletion of AMPA receptors by lysosomal degradation would decrease the size of the AMPA receptor pool available for cycling and activity-dependent reinsertion, which in turn would lead to reduced levels of synaptic AMPA receptors. Furthermore, it was demonstrated that GluR2 rapidly recycles back to the surface after endocytosis, and thus the intracellular accumulation level of GluR2 quickly reaches steady state at a low value (Lee, Simonetta et al. 2004). This suggests a predisposition to fill quickly a temporary gap of receptors in the membrane.

Several evidences such as ubiquitinated inclusions described in ALS patients (Bendotti, Atzori et al. 2004) and transgenic mice (Cheroni, Peviani et al. 2005) have suggested an abnormal proteolysis in the motor neuron degeneration of ALS which involved the ubiquitin proteasome system. However, other proteolytic pathways, such as endosome-lysosome system may be altered. Recently, proteomics studies in SOD1G93A mouse

spinal cord have shown increase in proteins associated with lysosomes (Lukas, Luo et al. 2006). These evidences suggest an altered mechanism of protein degradation by the lysosome that could alter the level of GluR2 subunit in the cytosol. Interestingly, internalized GluR2, but not other subunits, is sorted to endosomes-lysosomes in response to NMDA stimulation, suggesting that an overall glutamate receptor stimulus can contribute to increase the degradation of this subunit (Lee, Simonetta et al. 2004). This in turn could alter the finely regulated mechanisms of the intracellular trafficking of this protein leading to motor neurons being highly susceptible to glutamatergic stimulus. Further studies are necessary to confirm this hypothesis such as experiments of live imaging in SOD1 mutated culture cells to study the dynamic events that regulate the trafficking of this subunit between the membrane and the endosome/lysosome.

In addition, in this part of the thesis it has been analyzed the phosphorylated status of the GluR2 subunit with an antibody selectively directed to phosphorylated serine site 880. The phosphorylated protein is only present in the membrane as shown by the immunoband found exclusively in the LP1 fraction in western blot. This partially agrees with the higher immunofluorescence found near the motor neuron membrane in respect to the cytosol. However, no changes were detected in the motor neurons between the SOD1G93A mice at the presymptomatic stage and their non transgenic littermates. The phosphorylation status of the AMPA receptors is associated to a post-translational mechanism aimed to promote the internalisation of the GluR subunits into the cytosol in such way to maintain an uninterrupted recycling of these proteins between the cytosol and the postsynaptic membrane (Esteban, Shi et al. 2003; Boehm and Malinow 2005). Several kinases which are capable to finely regulate the surface expression and trafficking of the AMPA receptor subunits were described to interact post transcriptionally with GluR2; among them, the most important is PKC, a Ca^{2+} dependent enzyme (McDonald, Chung et al. 2001). There are evidences that the activity

of PKC is substantially increased in tissue from ALS patients, suggesting that alterations in intracellular free Ca^{2+} may be responsible for the increased activation also of this enzyme. An increased expression of PKC was reported in the spinal cord of ALS mouse model (Hu, Chernoff et al. 2003; Dave, Raval et al. 2005). Considering that the levels of the GluR2 subunits were found to be decreased in the cytosol and no phosphorylated protein was found in this compartment, I can not exclude that the increased activity of PKC might contribute to an increased internalisation of the protein favouring its pathway toward the degradation.

It is quite well established that motor neurons are more susceptible than other spinal neurons to toxicity mediated by AMPA receptors. It has been hypothesized that this selective vulnerability might result from differences in AMPA receptor subunit expression. For example, the presence of AMPA receptors lacking GluR2 or lacking edited GluR2 would confer an increased Ca^{2+} permeability, which in turn can lead to deleterious enhanced levels of intracellular calcium and cell death. Therefore, the decreased level of GluR2 showed in this study may be the cause of changes in homeostasis of the calcium in the motor neurons. The most important subcellular organelle able to store limited amount of calcium is the mitochondria. A series of evidences suggest a tight relation between altered glutamatergic stimulus and mitochondria dysfunction. An increased influx of calcium through Ca^{2+} AMPA permeable receptors induces a selective damage of the mitochondria in motor neurons (Carriedo, Sensi et al. 2000). On the other hand glutamate receptor alteration could be secondary to mitochondrial dysfunction induced by other factors such as the accumulation of mutant SOD1 (Liu, Lillo et al. 2004). In fact, a study carried out on mixed spinal cord cultures derived from the SOD1G93A transgenic mice showed an increased vulnerability of motor neurons to AMPA mediated glutamate toxicity (Spalloni, Albo et al. 2004). This was associated with enhanced reactive oxygen species

production, sustained elevations of intracellular calcium levels, and mitochondrial dysfunction (Kruman, Pedersen et al. 1999). In this respect, it is noteworthy that one of the earliest alteration in SOD1G93A mice is the vacuolization of mitochondria in motor neurons (Bendotti, Calvaresi et al. 2001).

However, other studies carried out on another model carrying G86R mutation in murine SOD1 did not find apparent changes in GluR2 immunostaining in spinal cord of these mice at both presymptomatic and symptomatic stage of the pathology (Morrison, Janssen et al. 1998). These mice develop an aggressive disease and only few days pass between the onset of symptoms and total immobility and death (Ripps, Huntley et al. 1995). This rapid development of the pathology, so different from the disease progression occurring in ALS patients and in SOD1G93A mice, renders this model quite different from the chronic course of the human illness.

With the progression of the disease, an increased reactive gliosis occurs in the spinal cord that becomes prominent at the end stage. I observed that GluR1, but not the other subunits, increased their expression in reactive astrocytes, while the GluR2 and 3 subunits become prominently expressed in microglia.

There is increasing evidence that functional glutamate receptors are not restricted to neurons, but also expressed in glial cells (Biber, Laurie et al. 1999; Noda, Nakanishi et al. 2000; Hirayama and Kuriyama 2001; Nakamura 2002; Taylor, Diemel et al. 2003; Hagino, Kariura et al. 2004; Christensen, Ha et al. 2006). Microglial cells are rapidly activated in response to even minor pathological changes so that they may be viewed as the cellular sensory element of brain pathology (Kreutzberg 1996). Moreover, microglia is known to be activated in glutamate receptor agonist-induced lesions, as demonstrated in hippocampal slice preparations where KA-induced neuronal excitotoxicity is associated with dose-dependent activation of glial cells (Akiyama, Tooyama et al. 1994; Abraham, Losonczy et al. 2001). Furthermore, neuronal death with a consequent release

of toxic factors can lead to the activation of microglia that in turn may exacerbate neuronal excitotoxicity (Moriguchi, Mizoguchi et al. 2003; Siao, Fernandez et al. 2003). The mechanisms underlying the upregulation of GluRs in reactive glial cells revealed in SOD1G93A mice is unknown. However, it has been shown that AMPA receptor stimulation mediates cytoskeletal alterations in microglia leading the cells from a resting to an activated state (Christensen, Ha et al. 2006). Other evidences have reported an altered expression of AMPA receptor subunits in the glial cells after excitotoxic stimulus which was associated with an enhancement of cytokines production and among them particularly increased was TNF α (Gottlieb and Matute 1997; Pellegrini-Giampietro, Gorter et al. 1997; Noda, Nakanishi et al. 2000; Hagino, Kariura et al. 2004). This is of particular interest given that the levels of this cytokine have been revealed to be augmented in the transgenic mice (Hensley, Floyd et al. 2002; Yoshihara, Ishigaki et al. 2002; Hensley, Fedynyshyn et al. 2003) and in CSF of ALS patients (Poloni, Facchetti et al. 2000). This suggests a possible relation between neuroinflammation and excitotoxic stimulus leading to synergic deleterious effect on the motor neurons.

Noteworthy, it has been demonstrated that the expression within motor neurons of mutated human SOD1 is the primary determinant of the disease onset of the transgenic mice, whereas diminishing selectively the mutant human SOD1 in the microglia has little effect on the early disease phase but sharply slowed later disease progression (Boillee, Yamanaka et al. 2006). This demonstrates a no cell autonomous mechanism in the developing of the disease and an important role during the progression of the disease could be ascribed to the activation of the microglial cells. Taken together, these results suggest that the activation of the microglia may initiate or contribute to motor neurons death by increasing their susceptibility to the toxic effects of glutamate.

CHAPTER 4

Study of the expression of glutamate AMPA receptor trafficking proteins in the spinal cord of SOD1G93A mice during the progression of the disease

4.1 EXPRESSION AND DISTRIBUTION OF AMPA RECEPTOR TRAFFICKING PROTEINS IN THE SPINAL CORD OF SOD1G93A MICE DURING THE PROGRESSION OF THE DISEASE EXAMINED BY IMMUNOFLUORESCENCE ANALYSIS AND BIOCHEMISTRY

4.1.1 HYPOTHESIS AND AIM

The previous results have shown a decrease of GluR2 in the motor neurons and particularly in the cytosol subfraction of ventral spinal cord from SOD1G93A mice at the presymptomatic stage, suggesting an alteration in the trafficking of these receptors.

Hence, I have examined whether changes in the expression and distribution of AMPA receptor trafficking proteins (ABP, PICK1 and NSF) occurs in motor neurons of SOD1G93A. Deregulation of AMPA receptors trafficking may influence the GluR subunits stoichiometry in the post synaptic membrane predisposing motor neurons to an uncontrolled influx of ions in the cytoplasm and altering the mechanism that regulate this homeostasis, leading to excitotoxicity.

I examined by immunofluorescence the protein expression of three important proteins involved in AMPA subunit trafficking (ABP, PICK1 and NSF) in lumbar spinal cord of SOD1G93A mice during the progression of disease in

respect to their non transgenic littermate. Moreover, I evaluated the levels and distribution of the AMPA receptor trafficking proteins in total homogenate and in subcellular fractions from lumbar spinal cord of SOD1G93A and non transgenic mice.

4.1.2 RESULTS

4.1.2.1 STUDY OF EXPRESSION OF GLUTAMATE AMPA RECEPTOR TRAFFICKING PROTEINS IN THE SPINAL CORD OF SOD1G93A MICE BY IMMUNOFLOURESCENCE

ABP staining:

In the spinal cord sections of control non transgenic mice, ABP resulted predominantly distributed in the motor neurons stained by FG and in the substantia gelatinosa (Fig 4, A; a,b). At high magnification a clear staining was revealed in the perikarya and in the dendrites of the motor neurons, including the distal part of the arborisation which was not stained by the FG (Fig 4, A; c,d). In transgenic mice at the presymptomatic (Fig 4, A; g,h arrow), symptomatic (Fig 4.1; c,d) and end stage (Fig 4.2, A; c,d) of the disease, I have not detected clear changes in ABP expression and distribution compared to control mice at the same age, except for a decreased staining in some vacuolized motor neurons (Fig 4, A; g,h arrow).

PICK1 staining:

In control mice PICK1 was mostly localized in the perikarya of the motor neurons and at lower level in the substantia gelatinosa (Fig 4, B; a,c). In SOD1G93A mice, this labelling increased diffusely in the perikarya of the neurons of the ventral horn and particularly in the motor neurons as shown by the increase colocalization signal of PICK1 with the FG at the presymptomatic stage (Fig 4 B; e-h). However, an increase signal was detected also in the dorsal horn of transgenic mice compared to control mice, at all stage of the disease examined (Fig 4 B; e-h , Fig 4.1, e-h, arrows , Fig 4.2 A; e-h). PICK1 was not found in reactive glial cells of SOD1G93A mice at the symptomatic and end stage (Fig 4.1, e-h, arrows , Fig 4.2 A; e-h).

NSF staining:

Analyzing NSF in control mice, I have revealed a labelling localized mostly in the perikarya of the motor neurons stained by FG (Fig 4, C; a,c). This labelling was increased selectively in the motor neuron cells as shown by an increase colocalization signal between NSF and FG at the preemtpomatic stage (Fig 4 C; e-h), but also at symptomatic and end stage of the disease of the transgenic mice (Fig 4.1, i-l, arrows, Fig 4.2 A; i-l), whereas, I did not detect differences in the dorsal horn of the transgenic mice (Fig 4 C; e,g , Fig 4.1, i,k , Fig 4.2 A; i,l). Furthermore, when I analyzed the distribution at the end stage of the disease, I detected a scattering signal exclusively for NSF that colocalized with some GFAP positive reactive astrocytes (Fig 4.2 B;h, arrows).

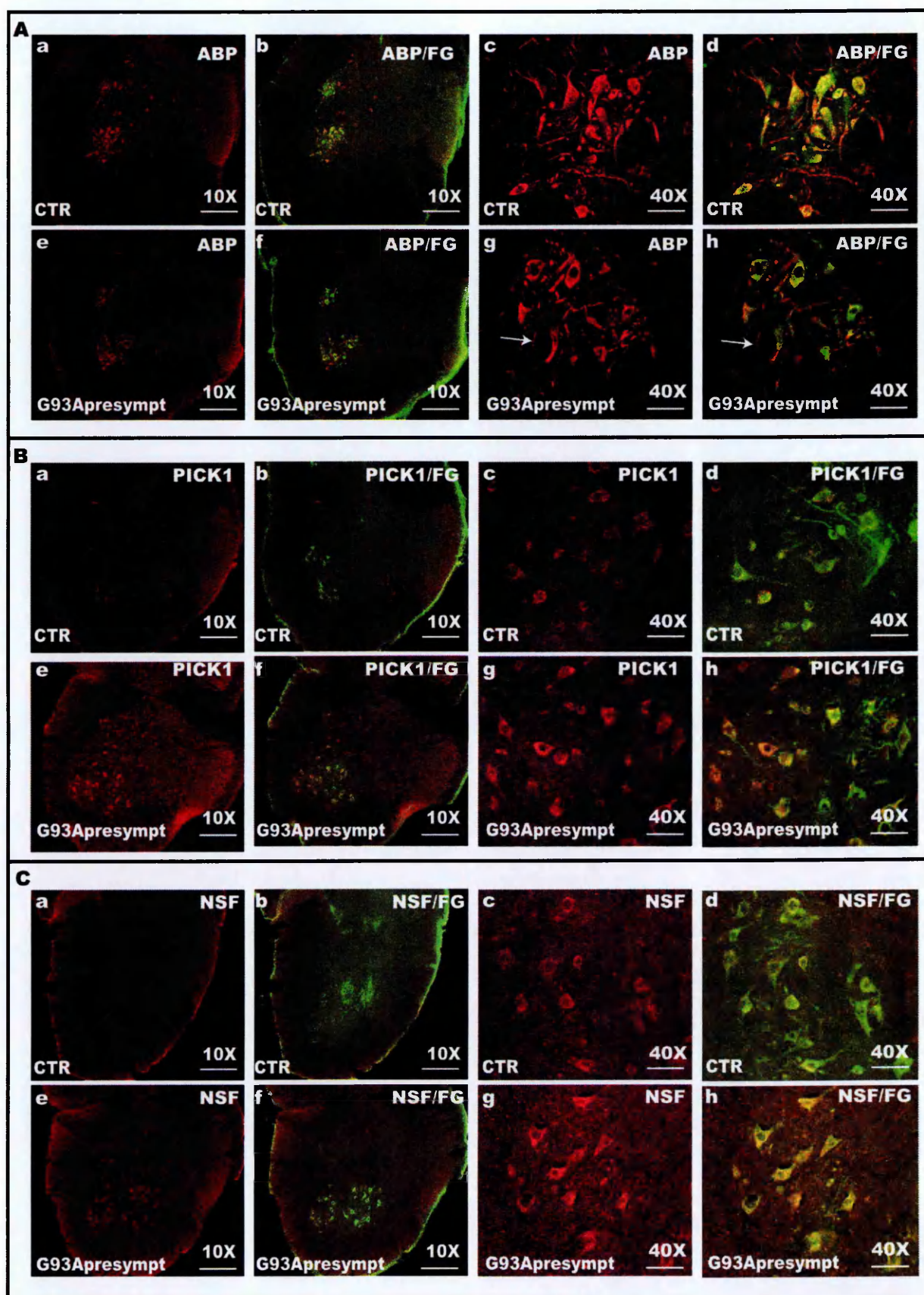


Fig 4: ABP, PICK1 and NSF immunofluorescence in lumbar spinal cord of SOD1G93A mice at the presymptomatic stage of the disease

At the presymptomatic stage of the disease, a marked immunoreactivity for ABP, PICK1 and NSF (red) is localized mostly in the motor neurons of ventral horn and in dorsal horn (A,B,C; a,c). In the motor neurons, stained by Fluorogold (FG, green), immunostaining for ABP is distributed in the perikarya and neurites (A; a,c), whereas both PICK1 and NSF are localized mostly in the cytosol of the motor neurons (B,C; a,c). No differences for ABP immunostaining are observed in the ventral and dorsal horn of SOD1G93A mice (A; a,e,c,g). Whereas, an increased labelling appears in the ventral region for both PICK1 and NSF (B,C; a,e,c,g), and also in dorsal region for PICK1 (B; a,e). In particular, we observed an increase of PICK1 and NSF immunostaining in the perikarya of motor neurons in the ventral horn (B,C; g). Scale bars: (A,B,C; a,b,e,f = 200 μ m); (A,C; c,d,g,h = 50 μ m).

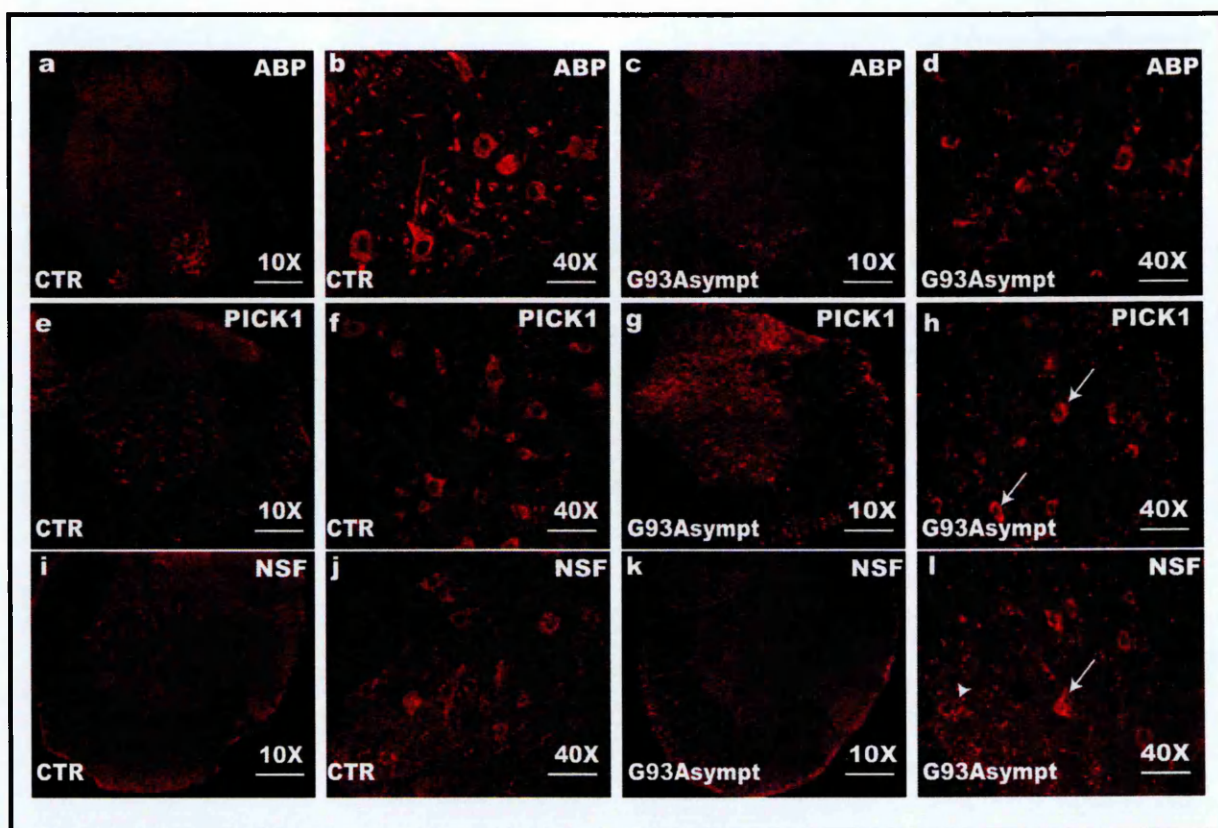


Fig 4.1: ABP, PICK1 and NSF immunofluorescence in lumbar spinal cord of SOD1G93A mice at the symptomatic stage of the disease

At the symptomatic stage of the disease, an increased immunoreactivity for both PICK1 and NSF (red) is showed in the motor neurons of ventral horn of SOD1G93A mice (h,l arrows), and in dorsal horn for PICK1 (g) compared to control mice (e,f,i,j). No differences for ABP immunostaining are observed in the ventral and dorsal horn of SOD1G93A mice (a-d). Scale bars: (a,c,e,g,i,k = 200 μ m); (b,d,f,h,j,l= 50 μ m)

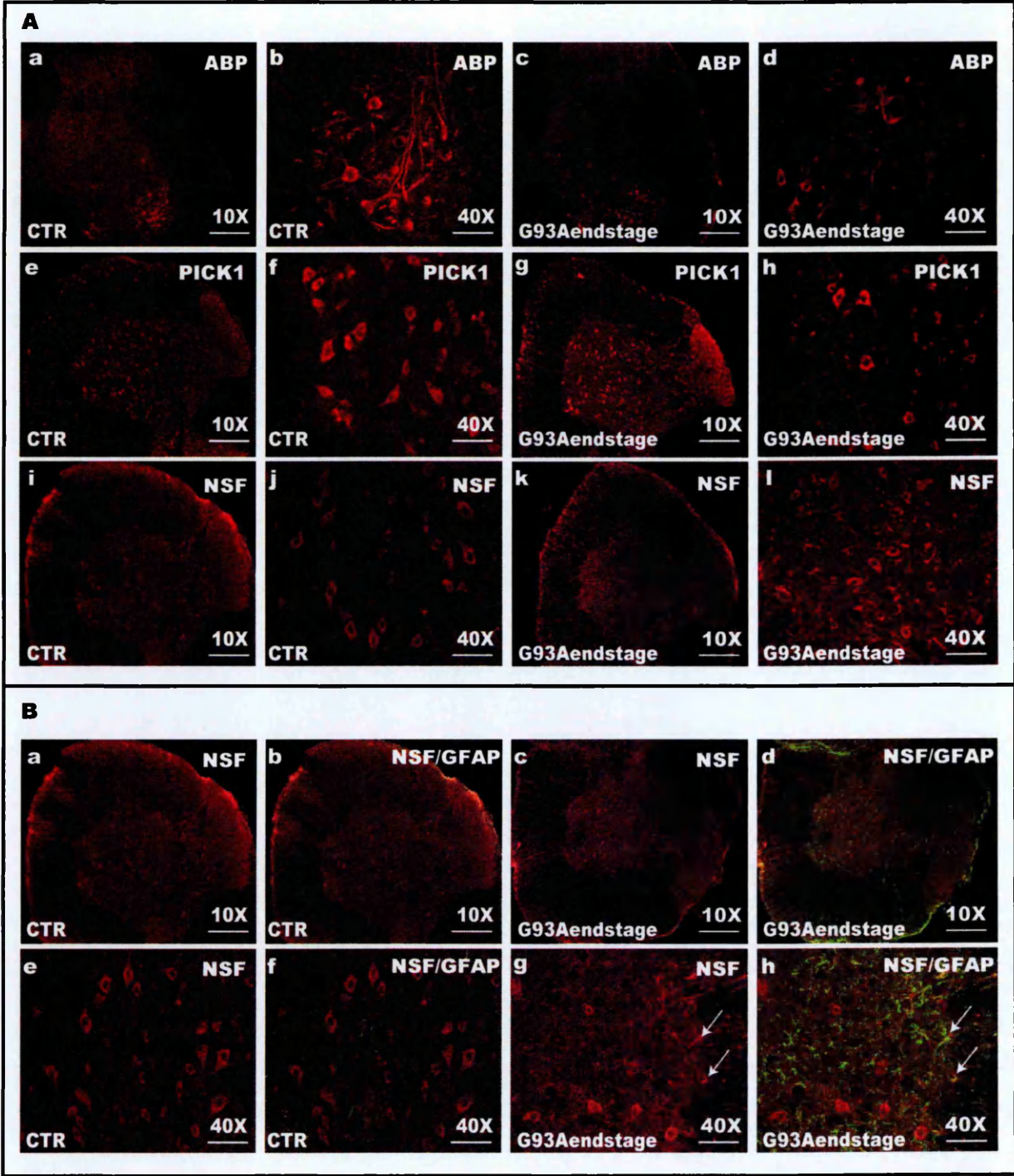


Fig 4.2: ABP, PICK1 and NSF immunofluorescence in lumbar spinal cord of SOD1G93A mice at the end stage of the disease

At the endstage of the disease, again an increased immunoreactivity for both PICK1 and NSF (red) is shown in the motor neurons of ventral horn of SOD1G93A mice (A; h,l) and in the dorsal horn for PICK1 (A, g), compared to control mice (A; f,j). Whereas, no differences for ABP immunostaining are observed in the ventral and dorsal horn of SOD1G93A mice (A; a-d). Furthermore, an increased scattering signal for NSF is revealed at this stage of the disease in the ventral horn of transgenic mice that colocalize with astrocytes showed in green (B; g,h, arrows) Scale bars: (A;a,c,e,g,i,k = 200 μ m); (A;b,d,f,h,j,l= 50 μ m); (B;a,b,c,d = 200 μ m); (B;e,f,g,h,= 50 μ m)

4.1.2.2 STUDY OF SUBCELLULAR DISTRIBUTION OF GLUTAMATE AMPA RECEPTOR TRAFFICKING PROTEINS IN THE SPINAL CORD OF SOD1G93A MICE BY BIOCHEMISTRY

The protein expression of AMPA receptor trafficking proteins ABP, PICK1 and NSF was investigated in the ventral and dorsal portion of the lumbar spinal cord of ALS transgenic mice at the presymptomatic stage in respect to age matched non transgenic mice. Tissues were obtained as total homogenate with the procedure described in section 2.3.1.1 or fractionated as described above in section 2.3.1.2. Two fractions were analyzed, a cytosol fraction, P3, and a synaptosomal membrane enriched fraction, LP1. The quantitative evaluation of the bands was carried out as described in section 2.3.1.3.

ABP protein:

The amount of ABP protein expressed in the ventral and dorsal spinal cord of SOD1G93A mice was tested at the presymptomatic stage. Analyzing the total homogenate and the two fractions, LP1 and P3, a detectable signal of ~80 KDa for ABP was revealed (Fig 4.3A, 4.4A)

A quantitative determination of the band optical density for the immunoblot on total homogenate has shown no differences between control and transgenic mice at the presymptomatic stage of the disease (Fig 4.3A). Similarly, no differences were found for LP1 and P3 fraction (Fig 4.4A). However, a p value for the LP1 fraction of $P=0.16$ close the significance has been detected.

PICK1 protein:

PICK1 showed a band of ~50 KDa in the total homogenate and in the subcellular fractionation of the ventral and dorsal spinal cord of SOD1G93A mice.

A quantitative determination of the band optical density has shown no differences in the total homogenate between control and transgenic mice at the presymptomatic stage (Fig 4.3B).

Analyzing the two fractions, LP1 and P3, only a detectable signal for PICK1 has been revealed in cytosolic fraction P3, which was not different from that found in the control mice (Fig 4.4B).

NSF protein:

The evaluation of NSF protein level expressed in the ventral and dorsal spinal cord of SOD1G93A mice revealed a ~75 KDa band in the total homogenate and in the two fractions analyzed (Fig 4.3C, 4.4D). A quantitative determination of the band optical density has shown no differences between control and transgenic mice at the presymptomatic stage of the disease for the total homogenate (Fig 4.3C). Interestingly, analyzing the two fractions LP1 and P3 at the presymptomatic stage no differences were found for the synaptosomal membrane enriched fraction LP1, whereas a significant increase was revealed for the cytosolic fraction P3 of transgenic mice compared to controls (Fig 4.4D).

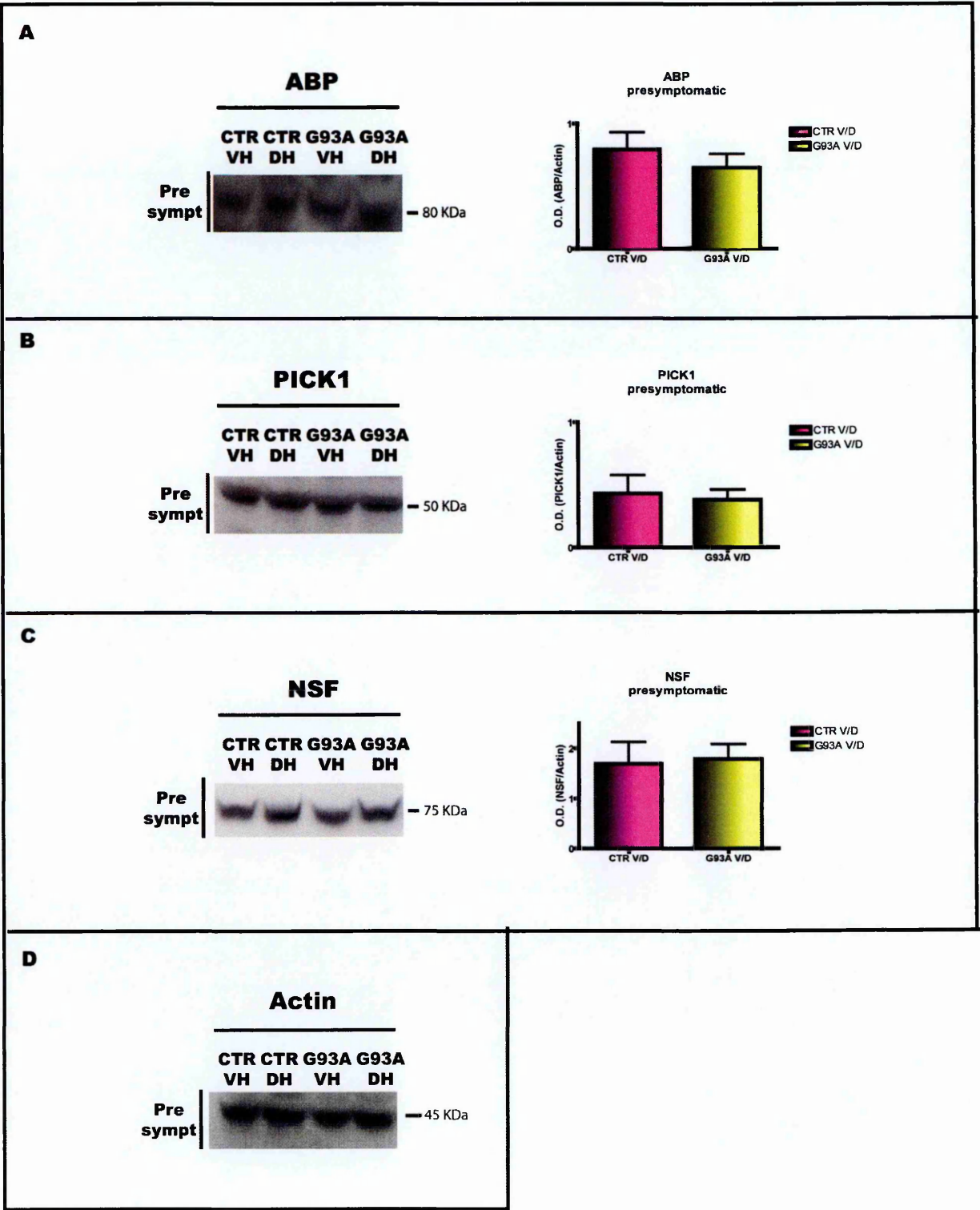


Fig 4.3: Western blot analysis of the expression of AMPA trafficking proteins in total homogenate of ventral and dorsal spinal cord of SOD1G93A mice

Lack of differences in ABP, PICK1 and NSF band intensity were observed in SOD1G93A mice at presymptomatic stage of the disease compared to controls at the same age (A,B,C). The graphics show quantitative evaluation of ABP, PICK1 and NSF (A,B,C) protein levels expressed as ratio between their band intensity and actin band intensity from the same blot (D). Each column shows the mean \pm S.E.M (n=7). Data analysed by Mann Whitney test.

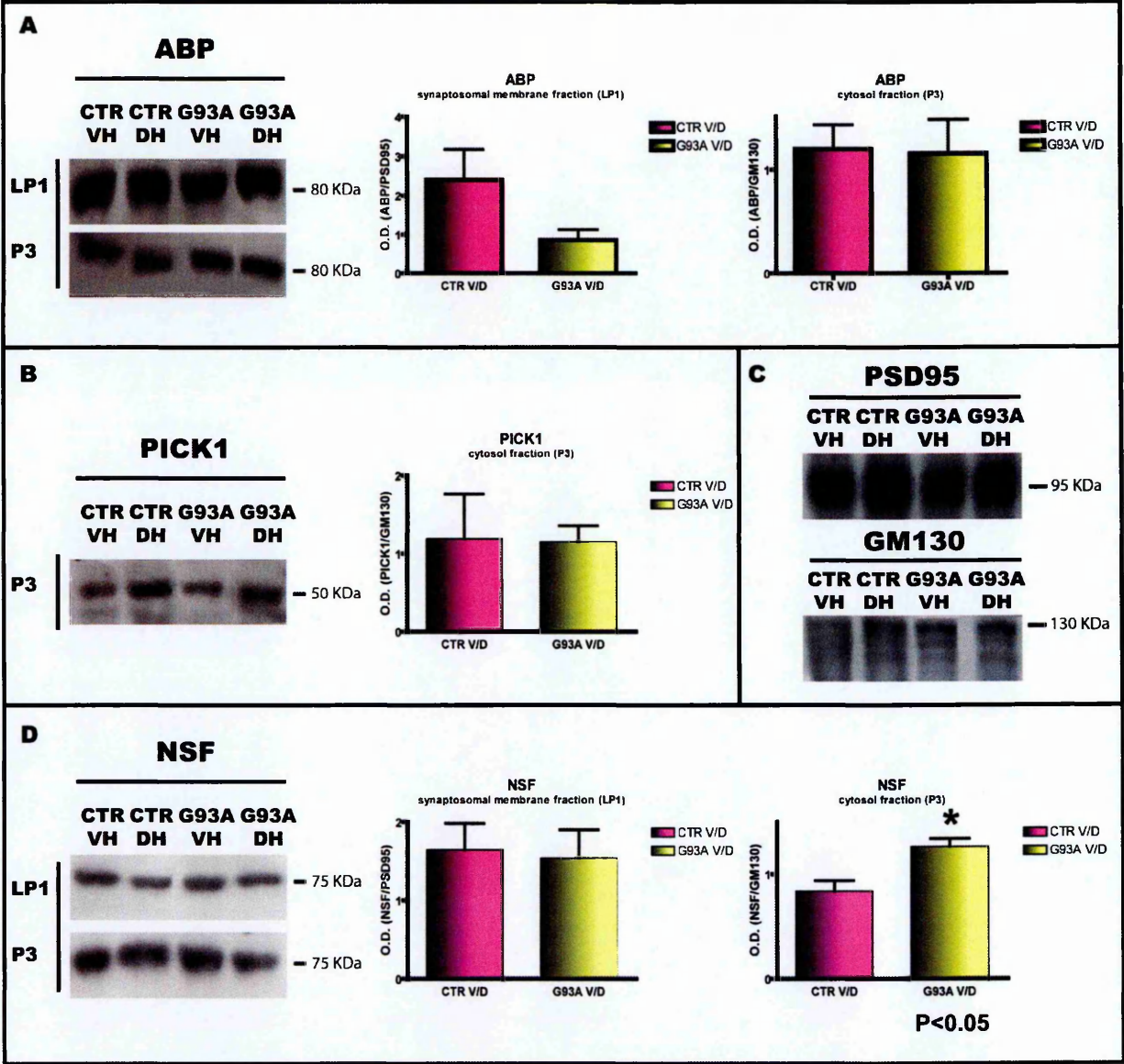


Fig 4.4: Western blot analysis of the expression of AMPA receptor subunits in subcellular fractionation of ventral and dorsal spinal cord of SOD1G93A mice

No evident differences in ABP and PICK1 band intensity, the latter revealed only in P3 fraction, were observed in SOD1G93A mice at the presymptomatic stage of the disease when compared to controls at the same age (A,B). A significant increase in NSF band

intensity was only found in the P3 fraction of transgenic mice compared to control (D). Graphics show quantitative evaluation of ABP, PICK1, NSF (A,C,D) protein levels expressed as ratio between their band intensity and PSD95 band intensity from the same blot for the LP1 fraction (B), whereas as ratio with GM130 for the P3 fraction (C). Each column shows the mean \pm S.E.M (n=7). Data analysed by Mann Whitney test.

4.2 DISCUSSION

The most important role of AMPA receptors is to mediate fast excitatory synaptic transmission in the nervous system. Changes in the responsiveness of postsynaptic AMPA receptors can regulate excitatory synaptic transmission. A possible mechanism underlying the modification of AMPA receptor activity is the change in the number of AMPA receptors in the postsynaptic membrane. In fact, it has been suggested that altering the trafficking of AMPA receptors to postsynaptic membrane leads to alterations in synaptic efficacy and that the prolonged activation is potentially neurotoxic (Lissin, Gomperts et al. 1998; Luscher, Xia et al. 1999; Shi, Hayashi et al. 1999; Lin, Ju et al. 2000; Luscher, Nicoll et al. 2000; Man, Lin et al. 2000; Turrigiano 2000; Wang and Linden 2000; Carroll, Beattie et al. 2001). In the neurons, AMPA receptors are clustered at postsynaptic sites (Craig, Blackstone et al. 1993; Molnar, Baude et al. 1993) with a significant proportion in the cytosol (Petrálie and Wenthold 1992; Martin, Blackstone et al. 1993; Molnar, Baude et al. 1993; Henley 1995; Hall and Soderling 1997). Changing the surface expression of AMPA receptors can result from a bidirectional rapid translocation from and to the intracellular pool. A tight cellular regulation of the trafficking of these receptors for a functional synaptic expression was characterized and associated with several cytoplasmic proteins such as GRIP/ABP, PICK1 and NSF (Dong, O'Brien et al. 1997; Kennedy 1997; Ziff 1997; Nishimune, Isaac et al. 1998; Osten, Srivastava et al. 1998; Song, Kamboj et al. 1998; Srivastava, Osten et al. 1998; Li, Kerchner et al. 1999; Noel, Ralph et al. 1999; Wyszynski, Valtschanoff et al. 1999; Xia, Zhang et al. 1999; Man, Lin et al. 2000). They are involved in the regulation of post translational modification, targeting, trafficking, surface

expression and anchoring of AMPA receptors. In this part of the thesis the distribution and expression of these three important proteins was tested. The main findings of this study are the changes found for PICK1 and NSF. A diffuse increased staining in the neurons of the grey matter was detected already at the presymptomatic stage of the disease for PICK1 in the lumbar spinal cord of the transgenic mice, which persists during the progression of the disease in the remaining neuronal cells. Whereas a selective increase staining in the motor neuron cells was found for NSF at presymptomatic stage of the disease and persist in the remaining motor neurons during the progression of the disease.

However, analyzing the total homogenate and the subcellular fractions of ventral and dorsal horn at the presymptomatic stage, no changes for PICK1 were revealed in P3 fraction. Whereas, no revealable signal was obtained in LP1 fraction, this in line with other studies showing the localization of PICK1 in perinuclear region, rough endoplasmic reticulum and Golgi apparatus (Staudinger, Zhou et al. 1995). This result is at variance with the immunofluorescence at the same stage; this is likely due to the sensitivity of this technique that is not able to reveal augmented level of this specific protein in a small portion of cells such as showed by immunofluorescence.

In contrast, a significantly increased level of NSF protein was found in the cytosolic fraction P3 of the transgenic mice, whereas no difference was revealed in the synaptosomal membrane fraction LP1.

Different roles were associated with these proteins. For PICK1, it was hypothesized that two mechanisms are involved in the trafficking of the AMPA subunits: the first suggests that the AMPA phosphorylation by kinases causes the recruitment of PICK1 to the synaptic AMPA subunits and then PICK1 promotes the internalization of AMPA subunit by clathrin-mediated

endocytosis. Alternatively, PICK1 can binds the AMPA receptors in the internal pool, stabilizing them and attenuating compensatory AMPA receptor insertion in the membrane (Xia, Chung et al. 2000).

NSF is involved in the disruption of multiprotein complexes with the AMPA receptor, such as those formed between the proteins of the postsynaptic density (Hanley, Khatri et al. 2002). In fact, it has been demonstrated that NSF regulates selectively the interaction between GluR2 and PICK1, disassembling PICK1-GluR2 complexes, resulting in an increased translocation of AMPA receptors to the cell surface (Hanley, Khatri et al. 2002). These suggest an opposite role for PICK1 and NSF in the finely regulated distribution of the AMPA receptor subunits between the postsynaptic membrane and the cytosol. Interestingly, NSF binds selectively GluR2, but not others subunits (Nishimune, Isaac et al. 1998; Beretta, Sala et al. 2005). Furthermore, NSF inhibits the targeting of GluR2 to lysosomes by promoting recycling of internalized GluR2 to the membrane. This hypothesis is supported by experiments showing that the lack of NSF is able to increase the sorting of GluR2 to the endosomes (Lee, Simonetta et al. 2004).

It is possible to speculate that the diffuse specific increase level of PICK1 in neurons of the transgenic mice may represent an epiphenomenon not related to the selective motor neuron degeneration. Furthermore, a lack of increased phosphorylation of the GluR2, which precedes the internalization of this subunit and the binding with PICK1, seems to suggest a non-determinant role of this protein in the regulation of the trafficking of GluR2 subunit during the progression of the disease. Instead, noteworthy is the specific increase level of NSF in the cytosol of the motor neurons, which may be considered an attempt to

counteract the altered degradation of GluR2 subunit and to stabilizing the steady state level of GluR2 in the membrane.

Another interesting result was the increased expression of NSF in the hypertrophic astrocyte cells at the end stage of the disease of transgenic mice. So far, in the literature this protein was characterized in neurones (Nishimune, Isaac et al. 1998; Osten, Srivastava et al. 1998; Song, Kamboj et al. 1998; Noel, Ralph et al. 1999; Hanley, Khatri et al. 2002; Beretta, Sala et al. 2005) without any detailed analysis in the glial cells. This is the first evidence of the presence of NSF in glial cells in a pathologic environment. Other studies are necessary to explain what is the role of this alteration in this pathology.

CHAPTER 5

GENERAL DISCUSSION

Amyotrophic lateral sclerosis is the most common form of motor neuron disease. There is not a cure or treatment today that halts or reverses this pathology. Studies carried out on post mortem human tissues and mouse models of ALS have revealed the involvement of a large number of different intracellular pathways and alteration of several biochemical systems in motor neurons and surrounding glial cells. Even so, the general picture of the disease and of its pathogenesis still results uncertain, since almost every alteration found can be the consequence or cause of the others. Relevant in order to understand the disease mechanism and development of treatments are the early events that characterize this pathology. An important breakthrough occurred with the discovery of mutations in the gene SOD1 as a primary cause of some forms of familial ALS. Since this relevant discovery, *in vitro* and *in vivo* models were developed and have helped us to begin understanding mechanisms involved in motor neuron death at the early stage of the disease and allowed us to test potential new therapies. Among the different model, the most used is the transgenic mice expressing some of the SOD1 mutants discovered in ALS patients, which display symptoms and neuropathological features that closely resemble the human disorder.

ALS clinical symptoms and neuropathological characteristics showed by patients affected by familial ALS are very similar to those occurring in sporadic case of the disease. This suggests that selective motor neuron death can be triggered by different events that, however, lead to a common intracellular pathway of death. Thus, the comprehension of the death processes that lead to the pathology in FALS mice will also be useful to the treatment of sporadic form of ALS.

To date, only one drug is approved by FDA for ALS, Rilutek® (Riluzole), which is able to slow down the progression of ALS, but with a modest effect. This drug has been demonstrated to be able to interact with the glutamatergic stimulus. This suggests that an excitotoxic event could represent a crucial point in the developing of the disease.

In this study, I have used the SOD1G93A transgenic mice to investigate the role of excitotoxicity in the etiopathology of the disease. In particular, I have studied possible alterations in the trafficking of the AMPA receptor subunits and the main proteins that regulate these dynamic events, considering these alterations as a potential trigger or contribution to motor neuron degeneration.

There is substantial evidence that decreased levels of GluR2 generate AMPA receptors highly permeable to calcium ions leading to excitotoxic processes (Hollmann, Hartley et al. 1991; Hume, Dingledine et al. 1991; Verdoorn, Burnashev et al. 1991; Burnashev, Zhou et al. 1995; Kuner, Beck et al. 2001). The selective AMPA antagonist NBQX showed protective effects on MND mice, significantly improving the motor function scores, and on SOD1G93A mice, prolonging their survival (Mennini, Cagnotto et al. 1999; Van Damme, Leyssen et al. 2003). The same effect on SOD1G93A mice was obtained in a study with another AMPA antagonist RPR 119990, which also improved the grip muscle strength of these ALS mice (Canton, Bohme et al. 2001). Moreover, our group has demonstrated that a new 2,3 benzodiazepine, ZK 187638, which displays non competitive AMPA antagonist properties, led to improvement of symptoms and a significant prolongation of the life span of SOD1G93A mice (Tortarolo, Grignaschi et al. 2006). These promising results indicate that blockade of AMPA-mediated glutamatergic stimulus could be an important therapeutic target. It is interesting to note that one of the earliest

neuropathological features observed in the motor neurons of SOD1G93A mice is represented by mitochondrial swelling which is particularly sensitive to altered calcium homeostasis (Bendotti, Calvaresi et al. 2001). Furthermore, increased vulnerability of motor neurons expressing SOD1G93A to glutamate toxicity by AMPA receptors was described in cultured cells (Spalloni, Albo et al. 2004).

It is still unclear, however, how the AMPA receptors, and in particular GluR2, can be regulated to determine the selective vulnerability of spinal motor neurons. A possible abnormality of the mechanisms regulating the bidirectional trafficking of the AMPA subunits between the cytoplasm and the postsynaptic membrane may represent a new important target that if compromised may lead to motor neuron degeneration.

About this topic, reduced levels of GluR2 protein, but not mRNA, have been recently documented by our group (Tortarolo, Grignaschi et al. 2006). In this thesis, I have extended those results demonstrating that a significant reduced levels of GluR2 was present in the cytosol of the motor neurons of the transgenic mice (Chapter 3), whereas unchanged levels of the protein was revealed in the postsynaptic membrane of transgenic mice when compared to control mice. It is possible to speculate that a reduced level of GluR2 is associated to increasing degradation activity of lysosomal machinery, the only degradation pathway so far characterized for the AMPA receptors. Interestingly, an altered lysosomal activity was documented in ALS patients and transgenic mice (Kikuchi, Yamada et al. 2003; Lukas, Luo et al. 2006) that could support this hypothesis. Furthermore, it was also demonstrated that an increased stimulation of glutamate receptors is able to augment the degradation by lysosome of the AMPA receptor subunits (Lee, Simonetta et al. 2004). This

corroborates the evidences in ALS patients of a marked decrease in the glutamate uptake in synaptosomes from spinal cord and motor cortex (Rothstein, Martin et al. 1992) and a massive loss of EAAT2 immunoreactivity in the same areas of the nervous system affected pathologically (Rothstein, Van Kammen et al. 1995). Furthermore, western blot analysis of the spinal cord total proteins of transgenic mice carrying G85R and G93A SOD1 mutant or transgenic rat model mutated in G93A have also shown a decline of GLT-1 expression (Bruijn, Becher et al. 1997 , Bendotti, Tortarolo et al. 2001, Howland, Liu et al. 2002). Taken together these results suggest an increase in the level of glutamate in the synaptic cleft available to stimulate glutamate receptors.

Despite a lack of change revealed in the postsynaptic membrane in this thesis, where the AMPA receptors are functionally active, there are evidences in literature that the trafficking of the AMPA receptors between the postsynaptic membrane and the cytosol is finely regulated, suggesting that a perturbation of this equilibrium can lead to deleterious effects in the glutamatergic stimulus (Carroll, Lissin et al. 1999; Luscher, Xia et al. 1999; Beattie, Carroll et al. 2000; Ehlers 2000; Lin, Ju et al. 2000; Malinow, Mainen et al. 2000; Man, Lin et al. 2000; Wang and Linden 2000; Carroll, Beattie et al. 2001).

It is also known that the presence of the Q/R edited form of GluR2 in the molecular structure of AMPA receptors determines the calcium impermeability of the channel. As outlined in the introduction to this chapter, changes in GluR2 edited form with an unedited form in the motor neurons can results in increased calcium permeability of AMPA receptors and might lead to pathological condition in ALS, determining or contributing to motor neuron degeneration. Furthermore, it was demonstrated that the unedited form at the Q/R site is much

more efficiently transferred to the membrane than the edited form (Greger, Khatri et al. 2002; Greger, Khatri et al. 2003). For instance, the efficiency of this editing is significantly reduced in the spinal ventral grey matter of individuals with ALS compared to control groups (Takuma, Kwak et al. 1999; Kawahara, Ito et al. 2004). However, these data were not confirmed by other groups and in a rat model of ALS carrying mSOD1, the GluR2 mRNA was completely edited in motor neurons. Other indications of a deregulated trafficking of the AMPA receptor revealed in my thesis were the increased expression of NSF in concomitance with the GluR2 downregulation in the motor neurons of transgenic mice. Increasing level of NSF correlates with an increased delivery of AMPA receptors at the cell surface. Hence, it is possible to suppose that an increase level of this protein is an attempt to stabilize the GluR2 subunit in the membrane as a consequence of a decrease availability of GluR2 in the cytosol of the motor neurons. Again, NSF seems to inhibit also the targeting to lysosomes by promoting recycling of internalized GluR2 to the membrane (Lee, Simonetta et al. 2004), increasing in strength the hypothesis of an attempt to stabilize a pathologic condition.

Abnormal Ca^{2+} influx through AMPA receptors in neurons can also determine activation of stress pathways, which in turn, may contribute to activation of death processes. Among them, one of the most important is linked to the p38MAPK, a mitogen activated kinase, which our group demonstrated to be activated (phosphorylated) in SOD1G93A mice (Tortarolo, Veglianesi et al. 2003). On the other hand, the action of phosphorylated p38MAPK was also correlated to altering the AMPA receptor composition. In fact, it has been demonstrated that damage processes can be mediated by AMPA receptors through p38MAPK signalling after an excessive Ca^{2+} influx through glutamate

receptors (Rivera-Cervantes, Torres et al. 2004; Torres, Chaparro-Huerta et al. 2006).

With the progression of the disease, a massive activation of glial cells, both astrocytes and microglia, occur in the ventral horn of lumbar spinal cord of SOD1G93A mice. The results reported in this thesis showed that GluR1, 2 and 3 expression is remarkably increased also in the glial cells during the progression of the pathology (Chapter 3). So far, the mechanism underlying the upregulation of GluR in reactive glial cells revealed in SOD1G93A mice is not completely known. However, it was reported that an altered expression of AMPA receptor subunits occurs in glial cells after an excitotoxic stimulus which was associated with an enhancement of production of cytokines such as TNF α (Gottlieb and Matute 1997; Pellegrini-Giampietro, Gorter et al. 1997; Noda, Nakanishi et al. 2000; Hagino, Kariura et al. 2004). This may account for increase levels of this cytokine in the transgenic mice (Hensley, Floyd et al. 2002; Yoshihara, Ishigaki et al. 2002; Hensley, Fedynyshyn et al. 2003) and in CSF of ALS patients (Poloni, Facchetti et al. 2000). Furthermore, published results obtained in our laboratory showed that TNF α receptors are over expressed in the motor neurons of presymptomatic stage and in the microglial cells during the progression of the disease in SOD1G93A mice (Veglianese et al. 2006).

In addition, inhibition of AMPA receptors by CNQX antagonist prevented the toxic effects on the motor neurons of activated microglia stimulated by lipopolysaccharide or IgG immune complexes from patients with ALS (Zhao et al. 2004), suggesting once again a link between excitotoxicity and neuroinflammation .

In conclusion, this study demonstrates an altered composition of glutamate AMPA receptors in motor neurons of SOD1G93A mice. In particular, decreased levels of GluR2 in the motor neurons may increase the susceptibility of these cells to the physiologic glutamate stimulus. Since the lack of GluR2 was observed mostly in the cytosol, this indicates an altered trafficking of the receptor subunit which might be due to an increased degradation of the protein by the lysosomal pathway. Interestingly, we observed in the motor neurons at the presymptomatic stage an increase level of NSF, whose activation results in augmented delivery of AMPA at the membrane. So, the increase of NSF could be an attempt to stabilize the remaining GluR2 AMPA receptor at the membrane (Fig 5.1).

Furthermore, the vulnerability of these neurons, in respect to glutamate-induced degeneration, is likely exacerbated by increased expression of GluR subunits in the glial cells during the progression of the disease. These cells might be able to release factors that are toxic for the motor neuron cells.

Ongoing experiments: to confirm the relevant role of these proteins in transgenic mice, a treatment with antagonist peptide that interferes with the interaction of NSF in C-terminal sequence of GluR2 in vivo could represent a good possibility to define the role of these proteins in ALS, and as therapeutic targets to develop potential drugs for ALS patients.

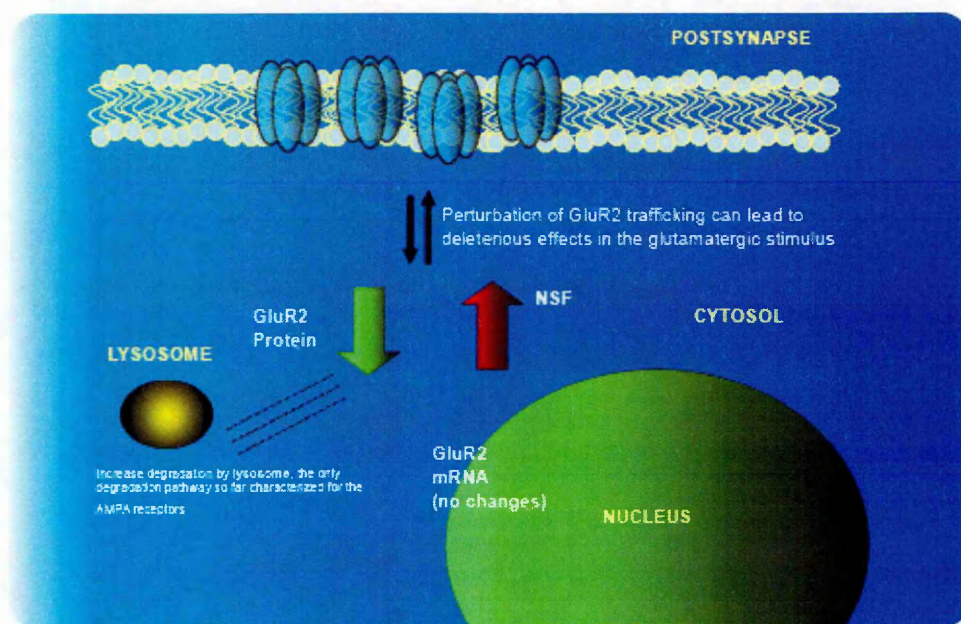


Fig 5.1: Cartoon of the results obtained

NSF protein increase in the cytosol (red arrow)

GluR2 protein decrease in the cytosol (green arrow)

The hope is that the results obtained in this study may contribute to the development of future new therapeutic strategies, which can help people affected by this devastating pathology.

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